The effects of anoxia and hypoxia on cerebral metabolism.

Wise, Helen

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THE EFFECTS OF ANOXIA AND HYPOXIA ON CEREBRAL METABOLISM

Submitted by Helen Wise

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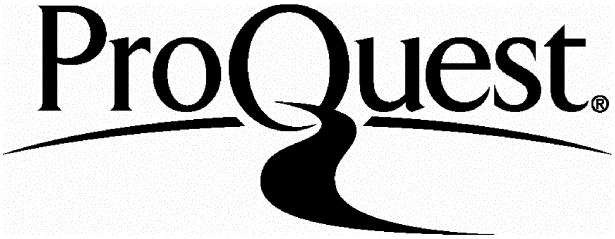

TO MY PARENTS

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CONTENTS

ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AEC	Adenylate energy charge
BSA	Bovine serum albumin
BuChE	Butyrylcholinesterase
CBF	Cerebral blood flow
CMR_{O_2}	Cerebral metabolic rate for oxygen
COMT	Catecholamine-O-methyltransferase
DA	Dopamine
FFA	Free fatty acid
GABA	γ-Aminobutyric acid
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine
HVA	Homovanillic acid
LDH	Lactate dehydrogenase
NA	Noradrenaline
PCr	Phosphocreatine
P_{O_2}	Partial pressure of O_2 (oxygen tension)
P_{AO_2}	Partial pressure of O_2 in arterial blood
P_{VO_2}	Partial pressure of O_2 in venous blood
SDAT	Senile dementia of the Alzheimer's type
SDH	Succinate dehydrogenase

SUMMARY

Synaptosomes (isolated nerve-endings) have been prepared from the rat cerebral cortex according to Bradford et al. (1975) and used to study the effects of anoxia and hypoxia on cerebral metabolism.

Experiments using a Rank oxygen-electrode have shown that synaptosomes behave in a manner reflecting the presence of the intra-synaptosomal mitochondria when placed in a mitochondrial incubation medium, e.g. (1) they respond to the addition of ADP by increasing O_2 consumption when supported by exogenous respiratory substrates such as glutamate and succinate, but not to the extent expected of pure mitochondria, (2) hypotonic shock reduces O_2 consumption and does not improve the ADP response, (3) ADP stimulation is blocked by oligomycin which can be overcome by DNP, and (4) the presence of a Na^+, K^+-ATPase in the synaptosomal limiting membrane is indicated by increased O_2 consumption on addition of Na^+ which is reversed by ouabain.

Synaptosomes behave in a similar manner in a hypoxic medium ($[O_2] > 4\mu M$) as in an air-saturated Krebs phosphate medium, e.g. (1) a critical O_2 concentration of 4μM was found where O_2 consumption became dependent on the P_{O_2} below this concentration, regardless of the initial P_{O_2}, and (2) glucose and bovine serum albumin stimulate O_2 consumption though by different, independent means. Any pre-incubation of synaptosomes should be carefully considered as should the composition of the medium since they will determine the rate of O_2 uptake.

There is no evidence that anoxia and hypoxia alters the accumulation and distribution of long-chain fatty acids and the increased respiration and glycolysis in a Ca^{2+}-free medium indicates the dependency of cerebral metabolism on the Ca^{2+} concentration in hypoxia. It is concluded therefore that other oxygen-dependent

processes such as monoamine synthesis, are responsible for the behavioural and functional alterations occurring at degrees of hypoxia too mild to affect ATP production.

CHAPTER 1

INTRODUCTION

Chapter 1 INTRODUCTION

1. OXYGEN AND LIFE

The importance of oxygen, water, and food to the animal organism is fundamental and of these three basic essentials for the maintenance of life, the deprivation of oxygen leads to death most rapidly.

If cerebral O_2 delivery is arrested, consciousness is lost in a few seconds. Zander (1975) determined the O_2 solubility in human cerebral cortex for both cerebral white and grey matter. Assuming a mean P_{O_2} of 30mmHg, the so-called "O_2 store time" will be 3 sec for grey matter and 14 sec for white matter. From this calculation it can be seen that the amount of O_2 physically dissolved in brain tissues and water would be sufficient to sustain cerebral metabolism at its normal level for a short period only. However aerobic metabolism of the brain must continue at its normal rate in order to preserve mental functions. No condition is known in which normal mental function is maintained in spite of a subnormal O_2 uptake (Lassen, 1959).

Ultimately the survival of the entire organism will depend upon its individual components, and under various conditions, any one of many different tissues may be the weakest link. However consideration of the maximum length of time that various tissues can be revived following acute anoxia: brain cortex, 5 min; heart, 10 min; liver and kidney, approx. 1 hr; and skeletal muscle, 8 hr in the resting state, it is clear that the brain shows considerable vulnerability to O_2 lack (Tenney & Lamb, 1965).

However, not only is O_2 lack threatening to life but so also is an O_2 excess. Acute exposure of mammals to O_2 at high pressure produces convulsions and repeated exposure of rats to O_2 at 4 atm.

often results in permanent damage to the CNS (Haugaard, 1968). At the cellular level for example, pyruvate oxidation in brain homogenates incubated at 5 atm. for 30 min is depressed to 20-33% of control values at 1 atm. (Haugaard, 1968).

O_2 is itself potentially toxic to all respiring cells due to the production of O_2^- and H_2O_2 from the biological reduction of O_2. These relatively minor products are themselves capable of yielding more lethal free radicals by the Haber-Weiss reaction =

$$H_2O_2 + O^-_2 \rightarrow OH\cdot + OH^- + O_2^*$$

O_2 toxicity is normally held in check by a balance among the rates of formation and destruction of these reactive forms of O_2 which may cause peroxidation of lipids and thereby initiate irreversible membrane damage. If the Haber-Weiss reaction is of real importance in vivo, then the adequacy of the defences against it may have far-reaching implications in the etiology of specific diseases and the senescence of aerobic living systems (Kellogg& Fridovich, 1975).

2. DEFINITIONS

In the literature one finds that the term hypoxia is readily interchanged with the following terms: hypoxaemia, anoxia, anoxaemia, anaerobiesis, cyanosis and ischaemia. This leads to confusion regarding the precise nature of the condition being studied. The following definitions have been taken from Siesjö (1978).

Anoxia	Absence of O_2.
Hypoxia	A reduction in available O_2 to levels that are insufficient to maintain function, metabolism or structure.
Ischaemia	A reduction of CBF to levels that are insufficient to sustain normal cerebral function, metabolism or structure.

These groups can be subdivided further:

Arterial hypoxia - hypoxic hypoxia (a reduction in O_2 content of blood).
- anaemic hypoxia (a reduction in haemoglobin content of blood).

Venous hypoxia - equivalent to ischaemia (reduced CBF).

Ischaemia - generalized (affects the brain as a whole).
- regional

Generalized/regional ischaemia - incomplete (CBF is reduced but has not ceased altogether).
- complete.

3. CEREBRAL O_2 SUPPLY

Arterial blood contains approximately 20 vol % of O_2 and only 0.3 vol % of this is in simple solution, the remainder is combined with haemoglobin (Schneck, 1971). The amount of O_2 reaching the brain will depend primarily on two factors, (1) the O_2 content of the blood, and (2) the cerebral blood flow rate (CBF).

The various conditions influencing the transport of O_2 from ambient air to blood will be considered in more detail later. The actual O_2 capacity of the blood is synonymous with the haemoglobin concentration and it is the O_2 affinity for haemoglobin that will in turn determine the amount of O_2 available to the tissues. The dissociation curve for oxyhaemoglobin is nearly horizontal at normal arterial O_2 tensions so that slight variations in P_{O_2} cause little change in the O_2 content of arterial blood and of cerebral O_2 delivery. There are two main factors influencing the position of the dissociation curve; pH and temperature. Acidosis displaces the dissociation curve to the right ('Bohr shift') such that the oxyhaemoglobin complex more

readily gives up its O_2.

In healthy young adults, CBF is 50-55ml/100g/min, thus the brain receives approximately 15% of the total cardiac output at rest (Lassen, 1959). The major portion of blood enters the cerebral circulation via the internal carotid arteries with a lesser amount coming from the vertebrobasilar system (Cohen, 1976).

CBF is under the influence of a variety of factors including autoregulation, intracranial pressure, blood viscosity and the pathology of the cerebral vessels. Over a wide range CBF is independent of changes in arterial blood pressure and remains constant until the mean arterial blood pressure falls to 60-70mmHg (Siesjö & Plum, 1973). When autoregulation, i.e. dilation of cerebral blood vessels in response to a fall in blood pressure, is lost due to generalised falls in systemic perfusion resulting in maximal vasodilation, the circulation then becomes pressure-passive.

The autoregulatory process is essentially independent of nerves with the observed contractions of the arteries being an automatic response to an increase in the distending pressure and vice versa.

O_2 is generally thought to act on the cerebral vessels via local mechanisms just as does CO_2. The influence of O_2 lack on CBF was considered to be due more to the accompanying acidosis which triggered vasodilation thereby increasing the supply of O_2 and metabolic substrates (Siesjö & Plum, 1973). Supported by other evidence, this led to a hypothesis being advanced to explain the chemical control of the cerebral circulation based upon the pH of the extracellular fluid close to the cerebral arteries. This was accompanied by the action of pH-sensitive chemoreceptors in the medulla to control P_{CO_2} in the brain by regulating ventilation. Since then however it has been realised

that although brain extracellular fluid pH may be one important factor influencing cerebral vascular resistance, this cannot be the sole controlling agent (Cameron, 1977).

Results of experiments using anaesthetised cats suggest that hypoxia induces pial arteriolar dilation indirectly - a process mediated via hypoxia of the neural cells rather than a direct action of the low P_{O_2} on vascular smooth muscle (Kontos et al., 1978).

ATP may also be involved in the control of local CBF because injection of ATP into the systemic circulation of baboons significantly stimulated CBF and cerebral O_2 consumption in a dose-dependent fashion (Forrester et al., 1975).

It has been suggested that adenosine, which dilates pial vessels in the rat, could also contribute to the regulation of CBF (Rubio et al., 1975). Similarly an extracellular K^+ concentration of 10mmol/ℓ in the rabbit is sufficient to produce dilation. This is part of a biphasic effect since at 50 mmol/ℓ, K^+ causes vasoconstriction and irreversible brain damage (Cameron, 1977).

The principle problem in determining which of the possible candidates is responsible for relating O_2 lack to CBF is in deciding upon the relative contributions of control from local factors and from the peripheral chemoreceptors. Currently there is increasing interest in the role of catecholamines in controlling CBF. Noradrenaline infused i.v. at 5μg/kg/min into rats increases CBF and CMR_{O_2}, the latter to 150% of control (Berntman et al., 1978). Adrenaline at a dose of 2μg/kg/min will produce similar responses and increasing the dose 4-fold increases CBF 2 to 6-fold. The increased CBF following adrenaline infusion is similar to that induced by hypoxia. It has been proposed that the CMR_{O_2} response is due to β-receptor stimulation and the CBF response is a constrictor α-adrenergic effect.

These results support an investigation by Kogure et al. (1979) using rats in which they deduced that the metabolic control of vascular tone requires an intact central adrenergic system. Because the metabolic influence on cerebral vessels is essentially proton mediated, they have suggested that the proton-sensitive site in the vascular wall requires an adrenergic input to function. In its absence, a fall in CMR_{O_2} does not cause vascular constriction and this leads to flow-metabolism uncoupling.

4. CELLULAR O_2 SUPPLY

The brain has a relatively high resting O_2 consumption and it therefore relies on a continuous supply of O_2. The force determining the rate of supply is the P_{O_2} gradient between capillary blood and tissue.

The first model of intercapillary O_2 transport was the Krogh cylinder model where a single capillary supplies a tissue cylinder by O_2 diffusion from the arterial to the venous end (Krogh, 1918/19). Krogh's derivation was based on the dual assumptions of unidirectional flow in parallel capillaries and of a temporally and spatially uniform O_2 consumption.

Lübbers (1965) concluded that the cylindrical model for tissue diffusion of O_2 was no longer valid since the P_{O_2} in neighbouring capillaries can be very different and therefore the P_{O_2} field between capillaries can be asymmetrical. This was later confirmed by Bicher et al. (1971) and Metzger & Heuber (1977) using microelectrodes which showed that brain O_2 levels varied sharply over short distances of a few microns. The range of P_{O_2} was for example 1-95 mmHg although when the microelectrode looked in one position, the P_{O_2} was remarkably constant. An arrangement of parallel capillaries with a reverse flow pattern better corresponds to in vivo conditions as an asymmetrical capillary model will allow better oxygenation.

These results are further supported by the observation that the apparent Km for O_2 of rat brain mitochondria is as low as 0.1μM (Clark et al., 1976) and that lowering of P_{VO_2} to 10mmHg did not alter the adenylate energy charge or the $NADH/NAD^+$ ratio. These results suggest that brain mitochondria may be sufficiently supplied with O_2 even when the driving capillary-tissue P_{O_2} gradient is of the order of 10mmHg (MacMillan & Siesjö, 1971). These results are incompatible with the calculations derived by Thews (1963) from analysis of the Krogh model which produced a "lethal threshold" value for P_{VO_2} of 12mmHg at which there was an immediate danger to life.

5. O_2 SENSOR?

When one considers that the brain is so very sensitive to hypoxia, it seems reasonable to assume the existence of an O_2 sensing mechanism within the brain. This assumption though is extremely difficult to verify since changes in P_{O_2} rarely occur in isolation from other chemical changes e.g. P_{CO_2} and pH which are known to be involved in homeostasis. Although peripheral chemoreceptors respond to lesser degrees of O_2 reduction, and discharge briskly when P_{O_2} values fall below 80mmHg, it has been seen that the hypoxic threshold for stimulating central homeostatic mechanisms lies close to that at which neurological deficits occur (Siesjö & Plum, 1973). The brain therefore functions with a low margin of error regarding O_2 availability.

Transient hypoxia (1 min) in rat brain shows that local P_{O_2} decreases rapidly to a new stationary level after 20 sec. Returning to air causes an overshoot which then returns to normal. Simultaneous measurement with microelectrodes of mean integrated action potential frequency showed after an initial increase, a sudden drop to zero. The overshoot on returning to air occurred 1-2 min after the P_{O_2} maximum (Erdmann et al., 1973). Critical P_{O_2} values here were defined

as the fall in action potential rate to <10/min and in tissue areas with a high initial P_{O_2}, the critical P_{O_2} value could be as high as 20mmHg compared to <0.5mmHg in districts with low P_{O_2}. In 1972, Duffy et al. suggested from experiments with hypoxic rats that when a "power failure" is imminent, neuronal activity is curtailed by a specific mechanism in order to conserve the slender stores of energy.

This point had actually been raised 30 years previously by Gurdjian et al. (1944) who demonstrated in morphinised dogs that the EEG showed an increase in amplitude on administration of 13% O_2, i.e. at a degree of hypoxia too mild to produce an increase in cerebral lactic acid or PCr levels. With 11.6% O_2 a definite slowing of the waves and a decrease in amplitude became evident. From this evidence they concluded that the occurrence of changes in EEG during hypoxia before the store of phosphate bond energy has begun to decrease, and while the glycolytic system is able to contribute energy for phosphorylation, suggests that the function of O_2 in relation to the EEG involves more than the supply of oxidative energy for phosphorylation.

This period of electrical silence in hypoxia had been considered by Bicher et al. (1971) to suggest (a) that neurones are very sensitive to their O_2 atmosphere and a slight drop is enough to silence them, or (b) this is an inhibitory reflex in which case a tissue P_{O_2} receptor should be theorized. This idea of active inhibition is one way of interpreting Bicher (1974) who found that lowering P_{O_2} locally in the right cortex can produce a period of "silence" in neurones firing in the contralateral or distal cortex.

The existence of a local O_2 sensor in brain tissue is also suggested by the use of computer simulation using known data on the physiological autoregulatory actions for O_2 consumption and blood flow rate as a function of tissue P_{O_2}. This computer simulation predicts

the occurrence of a "protective" effect in distal regions of the brain proximal to O_2 upsets via a decreasing rate of O_2 used or increased O_2 supply (Bruley & Hunt, 1974).

Bicher (1974) suggested four criteria to identify an O_2 autoregulation mechanism in brain tissue following a short period of anoxia; (1) short "reoxygenation time", (2) increase in CBF, (3) presence of an "overshoot", and (4) presence of a period of electrical silence paralleling the period of tissue P_{O_2} depression. Metzger & Heuber (1977) however consider that these results do not make it necessary to postulate the existence of a specific O_2 receptor within the cortex. They suggest that the action potentials obtained in response to hypoxia and hypercapnia can be explained on the basis of the de- and hyperpolarization responses seen in spinal cells. It may be that this change in membrane potential itself provides the basis for an O_2 sensor since a system of neurones whose rate of discharge depended on the level of tissue O_2 could function as chemical-electrical transducers. On many Aplysia neurones, small measurable changes in the intracellular P_{O_2} significantly affect the resting membrane potential of the somatic neuromembrane (Chalazonitis, 1977). The presence of oxyphores in membranes which are sensitive to O_2 might explain the direct action of O_2 in leading to permeability changes.

Assuming that a tissue O_2 sensor does exist within the brain raises the questions of what is it, where is it located, how does it exert its influence and is it the only monitor of hypoxic conditions? At the present time there are no simple answers to these questions. The candidates for the role of O_2 sensor as outlined by Jöbsis (1977) are summarized below:

1) Possible direct effect of O_2 on membranes: change in structure → permeability effects → membrane potential differences → action potentials.

2) Chemical mechanism: oxidation of structural lipids or lipo-proteins.

3) Neurotransmitter oxidases which have K_m's for O_2 of approx. 10^{-5}M (12mmHg or 1.5% O_2).

4) Cytochrome oxidase which handles the bulk of O_2 used in the cell.

5) Possibly increased adenosine or lactate or more likely H^+ as a mediator which would provide a common mechanism for normoxic regulation and for the more extreme hypoxic and anoxic states.

6. CEREBRAL O_2 UTILIZATION

Nearly 20% of the O_2 taken up by the whole body at rest is utilized by the brain. O_2 is required for two major functions: (1) for mitochondrial respiration where molecular O_2 is directly consumed in the final stage (cytochrome oxidase) of the electron transport system, and (2) for the synthesis and metabolism of monoamines (tryptophan hydroylase, tyrosine hydroxylase, dopamine β-hydroxylase and monoamine oxidase).

Less than 0.1% of O_2 consumed by the brain is utilized in direct synthesis and metabolism of NA, DA and 5-HT (Davis & Carlsson, 1973b). GABA degradation is also influenced by P_{O_2} as is also the cyclo-oxygenase enzyme responsible for the synthesis of the prostaglandins, thromboxane A_2 and prostacyclin.

The apparent K_m values for O_2 in some of these enzyme systems are: mitochondrial respiration, 0.1μM (Clark et al., 1976); tryptophan hydroxylase, 1.0mM (Kaufman, 1974); tyrosine hydroxylase, 0.5mM (Fisher & Kaufman, 1972); and cyclooxygenase, 5μM (Lands et al., 1978). All these values are taken from brain enzymes except for the latter which was determined for vesicular gland cyclooxygenase.

Because the bulk of O_2 consumed by the brain is involved in mitochondrial oxidative phosphorylation and thus energy synthesis, it is important here to mention briefly the energy dependent processes

occurring in the brain. These are neurotransmission, ATPase activity, transport (membrane and axonal), protein - nucleic acid - lipid synthesis, hydrolytic enzymes, and membrane structure.

In order to describe the energy status of the cell, the term "adenylate energy charge" (AEC) was introduced by Atkinson (1968). The AEC is defined as $[ATP] + 0.5\ [ADP] / [ATP] + [ADP] + [AMP]$. The AEC is relatively constant at 0.85-0.9 in a great variety of tissues and organisms and a fall in AEC is coincident with a rapid decline in cell viability (Drewes & Gilboe, 1973a). Although the concept of the AEC is a useful model, it is in fact a static concept and ignores other high energy metabolites such as PCr and GTP. It has been shown that the responses to the AEC of rat brain hexokinase, muscle phosphofructokinase and muscle pyruvate kinase depend on multiple factors which although including the total adenine nucleotide concentration, are not entirely dependent upon it (Levy & Duffy, 1977).

7. ANOXIA

7.1. Introduction

The term anoxia was subdivided by Barcroft (1920) to distinguish anoxic, anaemic and stagnant anoxia. Examples of these conditions are given below:

Anoxic anoxia - decreased availability of O_2 from the atmosphere
decreased respiratory movements
respiratory tract obstruction
interference with alveolar exchange
cyanotic congenital heart disease

Anaemic anoxia - haemorrhage
chronic anaemia
CO or CN intoxication.

Stagnant anoxia - shock
congestive heart failure
cardiac arrest
stenosis, occlusion of carotid or vertebral arteries extracranially.

Anoxia will lead to EEG changes within 8-15 sec irrespective of the form of O_2 lack. Albaum et al. (1953) noticed that in rabbits breathing N_2, function disappeared and there was an inexcitability of all measurable elements which was associated with only a moderate decrease in ATP, PCr and glycogen. Of particular importance was the observation that the loss of spontaneous electrical activity occurred before significant changes in chemical constituents could be detected. This loss of function before the energy stores are exhausted has been confirmed in anoxia (Siesjö & Plum, 1973) and in hypoxic conditions (Duffy et al., 1972; MacMillan & Siesjö, 1972; Bachelard et al., 1974).

7.2. The biochemistry of anoxia

The biochemical alterations associated with anoxia have been reported to be:

(a) A fall in PCr levels within 2 min decreasing further to less than 30% of normal after 5 min anoxia (Cohen, 1962).

(b) An increase in AMP by 75% reaching 10-fold values after 6 min. ATP fell by 50% and ADP increased slightly (Drewes & Gilboe, 1973a).

(c) The AEC decreased from 0.90 to 0.56 after 6 min and to 0.39 after a 10 min period of anoxia (Drewes & Gilboe, 1973a, 1973b).

(d) Cyclic AMP increased and cyclic GMP decreased (Gibson et al., 1978).

(e) Decreased capacity for lipid synthesis (Kosow et al., 1966), and significant increases in glycerophosphoethanolamine and phosphoethanolamine due to the mobilization of complex lipids (Drewes et al., 1977).

(f) Increased extracellular K^+ concentration (Hansen, 1977).

(g) The rate of glycolytic flux increased 5-fold after 1 min of anoxia possibly due to an increased activity of phosphofructokinase, hexokinase and glycogen phosphorylase (Drewes & Gilboe, 1973a).

(h) Changes in amino acid metabolism: uptake of histidine and lysine; efflux of glutamate and proline; increased tissue concentrations of five essential amino acids, methionine, histidine, leucine, lysine and valine; decreased threonine and phenylalanine; taurine, GABA and alanine increase with aspartic acid decreasing in concentration (Drewes et al., 1977). The similarity between amino acid changes observed for anoxic and ischaemic brains suggests that those amino acids which increased are probably derived from other cellular elements and not from the blood supply.

The various stages of anoxia with the corresponding chances of recovery are detailed below (taken from Drewes et al., 1973).

Early stage of anoxia

First 2 min. Increased rate of glycolysis.
Brain glucose falls.
ATP near normal.
AEC > 0.80.

Intermediate stage of anoxia

Brain no longer capable of generating adequate energy.
Control points of glycolysis still active - functional recovery if O_2 restored.

Rate of lactate production decreases - cerebral glycogen and glucose stores depleted.

Increased AMP from ATP.

AEC 0.39 after 10 min.

Late stage of anoxia

Loss of metabolic control.

Inability to recover function.

Still slow increase in lactate - substrate exogenous.

ATP falls to 5%)

Total adenylate 38% of control) these do not recover on reoxygenation

AEC 0.25)

8. HYPOXIA

8.1. Introduction

As defined previously, hypoxic hypoxia results whenever a fall in arterial P_{O_2} reduces O_2 transport to brain tissue to such a degree that functional, metabolic, or structural alterations occur. However in clinical medicine, uncomplicated hypoxic hypoxia is rarely observed but does occur in anaesthesiological accidents, lung disease and high altitude. The general symptoms of hypoxia include light-headedness, lassitude, headache, nausea and restlessness (Siesjö, 1978). More specific cerebral symptoms are those showing involvement of consciousness (mental confusion, stupor and unconsciousness), and those indicating impairment of visual discrimination, memory, or the performance of intellectual or motor tasks. A severe reduction in O_2 supply will produce generalized convulsions.

Cerebral dysfunction is one of the earliest signs of hypoxia in man. When inspired O_2 concentration falls from 21% to 16% there are errors in reading tests. At 12% O_2 one sees acute mountain sickness

and at 6-8% (P_{VO_2} 17-19mmHg) there is loss of consciousness (Courtice, 1941; Schneck, 1971; Davis et al., 1973).

8.2. The biochemistry of hypoxia

Although even relatively moderate degrees of arterial hypoxia lead to changes in cerebral function, metabolism and blood flow, P_{AO_2} in experimental animals can be lowered to 20-25mmHg without causing a clear perturbation of tissue concentrations of ATP, ADP and AMP (Duffy et al., 1972; MacMillan & Siesjö, 1972; Bachelard et al., 1974).

The sequence of effects occurring during a gradual reduction of P_{O_2} is as follows:

P_{AO_2}	Metabolic change.
<50mmHg	Increased intra- and extracellular lactate.
<35mmHg	Decrease in PCr.
20mmHg	Changes in ATP, ADP and AMP.

It should be pointed out here that clinically, loss of consciousness and major EEG abnormalities occur at P_{AO_2} 30mmHg, i.e. before changes in the adenine nucleotides (MacMillan & Siesjö, 1972).

The rate of entry of glucose into brain in the first few minutes of hypoxia is 300% of normal - this increased anaerobic glycolysis is due to activation of hexokinase and phosphofructokinase (Bachelard et al., 1974). The triggering factor(s) responsible for stimulating glycolysis in hypoxia is still unclear.

Another important factor influencing the response of the brain to hypoxia is the increase in CBF which occurs when P_{AO_2} falls below 50mmHg. If the P_{O_2} is reduced to minimal values of 22-23mmHg there is a 5-fold increase in CBF in rats (Jóhannsson & Siesjö, 1975). In fact the energy state is upheld in hypoxia only as long as an adequate perfusion pressure maintains a high and uniform blood flow through the

brain (BP > 120mmHg). [For factors influencing CBF, see Section 3].

Other biochemical changes associated with hypoxia are (a) an 80% increase in cGMP which occurs before significant increases in cAMP and lactate (Gibson et al., 1978), (b) the release of intracellular K^+ into the extracellular compartment of the brain (Bourke et al., 1978), and (c) increased concentrations of glycolytic and TCA cycle intermediates and NH_3 while glutamate and aspartate decreased in concentration (Yoshino & Elliott, 1970; MacMillan & Siesjö, 1972).

8.3. Homeostatic mechanisms in hypoxia

The maintenance of a normal, or near-normal, energy state in profound hypoxia indicates that powerful homeostatic mechanisms exist that secure an almost adequate supply of O_2 to the cells. One such mechanism is the increased CBF at P_{AO_2} < 50mmHg which may amount to several hundred percent of normal at extreme degrees of hypoxia (Jóhannsson & Siesjö, 1975).

Another mechanism proposed by Duffy et al. (1972) was a fall in CMR_{O_2} which would decrease energy demands. These experiments have not been confirmed (Bachelard et al., 1974; Jóhannsson & Siesjö, 1974) and it has been suggested that these results were due to a fall in body temperature since a fall of 3-4°C will reduce CMR_{O_2} by 15-20% (Hägerdal et al., 1975; Jóhannsson & Siesjö, 1975).

Up until 1978 it was generally believed that the maintenance of a normal energy status in hypoxia was due solely to an increased CBF since CMR_{O_2} remained constant (Bachelard et al., 1974, Jóhannsson & Siesjö, 1974). Further work by Berntman & Siesjö (1978a) indicated that CMR_{O_2} increased slightly in hypoxic starved animals. This paradoxical increase had in fact also been observed in fed animals and was suggested to reflect the influence of hypoxia on the activity of cerebral

catecholaminergic neurones. It is possible that hypoxia, like other stressful situations, may cause the activation of extrinsic and intrinsic catecholaminergic systems (e.g. adrenals) which would tend to enhance metabolic rate (Berntman & Siesjö, 1978b). Conformation of increased CMR_{O_2} in hypoxia comes from Berntman et al. (1979) who demonstrated that removal of the adrenal glands prevented this increase. Circulating catecholamines, and possible central catecholamine pathways, then can increase CMR_{O_2} to 180% of control depending on the breed of Wistar rats chosen for study.

8.4. Hypoxia and neurotransmission

Hypoxia will affect the neurotransmission process by influencing both neurotransmitter synthesis and the transmission process itself and these changes may possibly be the basis for the altered function at P_{AO_2} values that are unassociated with changes in ATP, ADP and AMP.

Firstly there are the changes in glycolytic and TCA cycle intermediates and their associated amino acids, e.g. glutamate, aspartate, GABA and alanine. Glutamate, aspartate and GABA are all involved in cellular metabolism and neurotransmission.

In 1967, Wood discovered that brain GABA levels were very sensitive to hypoxia with GABA levels increasing after exposure of rats to 8% O_2 for 10 min. It was suggested here that the increased GABA depresses nerve transmission thus conserving the available high energy compounds. Later this increase in GABA was shown to occur in a wide range of animals with a critical O_2 concentration of 7-8% (Wood et al., 1968) comparing well with the critical O_2 concentration of 8% for changes in lactate and PCr given by Gurdjian et al. (1949). Perhaps there is decreased utilization of GABA via the GABA shunt pathway of the TCA cycle potentiated by the continued production of GABA by anaerobic

glutamic acid decarboxylase (Wood et al., 1968).

Glutamate and aspartate - both putative excitatory neurotransmitters- decrease in hypoxia (MacMillan & Siesjö, 1972) and the incorporation of [U-^{14}C]-glucose into alanine increases due to the increased production of pyruvate (Yoshino & Elliott, 1970).

These changes have all been brought about principally by a mismatch between the rate of glycolysis and the rate of TCA cycle flux leading to an accumulation of pyruvate and a redox change (Siesjö, 1978). The changes observed in the metabolism of the catecholamine and indoleamine neurotransmitters however is brought about by the dependence of their enzyme systems on molecular O_2. As already pointed out (see Section 6) the hydroxylase enzymes are poor competitors for cellular O_2 with mitochondria. Neither tryptophan or tyrosine hydroxylase are fully saturated in the range of brain O_2 tensions (Davis et al., 1973). Even relatively mild hypoxia (10% O_2) significantly reduces the synthesis of the catecholamines DA and NA, and the indoleamine 5-HT (Davis & Carlsson, 1973b) showing the clear dependence of tryptophan and tyrosine hydroxylase on the supply of O_2 in vivo. These results correlate best with percent arterial saturation and not arterial pH or P_{O_2} (Davis & Carlsson, 1973a). Because both the synthesis and degradation steps are impaired by hypoxia, the brain monoamine levels appear unchanged even after 2 hr of hypoxia (Brown et al., 1975). For example, monoamine oxidase activity is inhibited by hypoxia leading to decreased levels of the metabolites HVA and 5-HIAA, DOPA decarboxylase and COMT are not affected (Brown et al., 1975).

Of significance here is the observation that hydroxylation rates were already reduced at degrees of hypoxia which did not affect the AEC and that the decrease in 5-HTP formation preceded the

accumulation of lactate in the brain cells (Davis & Carlsson, 1973a).

Since hypoxia appears to inhibit firing by dopaminergic neurones and to enhance firing by noradrenergic neurones, there is the possibility that the more prominant affect on DA turnover may be important for the preservation of energy in hypoxia (Carlsson, 1978). DA is also implicated in the hypoxia-induced changes in behaviour. Hypoxia has been found to disrupt reversibly the conditioned avoidance response (CAR) in rats, an affect reversed by treating the animals with DOPA or apomorphine (Brown et al., 1975). Since CAR is known to depend on intact catecholaminergic systems in the brain, it may be that inhibition of DA turnover is a regulatory mechanism functioning to inhibit in order to preserve energy in hypoxia (Carlsson, 1978).

After 24 hr at 10% O_2, hypoxic adaptation of monoamine synthesis is observed in rats (Davis, 1975). This adaptation correlates with changes in brain P_{O_2} rather than intraneuronal regulation of monoamine synthesis and can be traced to a shift in the oxyhaemoglobin dissociation curve which increases P_{O_2}.

Other influences on monoamine turnover are P_{CO_2} and stress. Synthesis of 5-HTP by tryptophan hydroxylase is influenced by P_{O_2} and not P_{CO_2} whereas DOPA synthesis by tyrosine hydroxylase is influenced more by P_{CO_2} than P_{O_2} - an affect which will become significant in the various forms of hypoxia (Carlsson, 1978). As already described, the rates of rat brain tryptophan and tyrosine hydroxylation are dependent on O_2, however during physical stress (curare-immobilization or electroshock), this O_2 dependency is lost (Davis, 1976). Davis suggests that these results are consistent with the hypothesis that during neuronal stimulation, an allosteric change increases the affinity of the enzyme for O_2 thus allowing greater synthesis despite limiting concentrations of this substrate.

The final neurotransmitter that has been studied in relation to hypoxia is ACh. Using an injection of $NaNO_2$ (this leads to 'anaemic hypoxia' by converting haemoglobin to methaemoglobin) Gibson & Blass (1976) demonstrated reduced incorporation of $[^2H_4]$-choline into ACh at doses of $NaNO_2$ which did not alter concentrations of ATP, ADP or the AEC. A similar result was obtained with hypoglycaemia with both hypoxia and hypoglycaemia increasing brain choline concentration. The total ACh only falls in severe hypoxia suggesting that a small pool of ACh with a high turnover rate is affected. Among the factors influencing ACh synthesis in brain are the concentrations of NAD and NADH which are both functions of the rates of carbohydrate utilization. Further experiments showed a 43% decrease in ACh synthesis from $[U\text{-}^{14}C]$-glucose accompanying hypoxia so mild that there were no significant changes in cerebral lactate or cAMP (Gibson et al., 1978). Although ACh synthesis from glucose is more sensitive to hypoxia than synthesis from choline, this effect cannot be accounted for by a change in glucose utilization.

The sensitivity of the neurotransmitter ACh to hypoxia is in accord with the physiological evidence that neurotransmission is more sensitive to hypoxia than is axonal conduction. Reducing the O_2 available to the isolated superior cervical ganglion results in increased glucose uptake and increased lactate in solution (Dolivo, 1974). This tissue is drawing all its energy from anaerobic glycolysis and this source of energy does not allow the maintenance of synaptic transmission. If glucose is withdrawn, synaptic transmission is lost in 2½ hr (irreversible) and axonal conduction after 24 hr (Nicolescu et al., 1966). Cholinergic transmission is therefore more sensitive to hypoxia and to low glucose concentrations than is axonal conduction.

All these results tend to express the idea that the functional effects of moderate hypoxia represent a failure in transmission rather than in energy.

9. ISCHAEMIA

9.1. Introduction

A discussion of ischaemia has been included in this introduction because of its interrelation with anoxia - a distinction which is not always made in the literature - and its clinical importance (e.g. stroke, heart failure). In fact it is probable that when hypoxia is severe enough to affect the energy state of the tissue then we are in fact looking at regional ischaemia rather than effects due to O_2 lack alone. Ischaemia differs from hypoxia in that there is not only an interruption of the supply of O_2 but also of exogenous substrates, especially glucose.

9.2. The biochemistry of ischaemia

Brief periods of cerebral ischaemia in animals produce morphological damage and a rapid decline in preformed and potential energy stores (Levy & Duffy, 1977). Complete cerebral ischaemia leads to a rapid fall in brain glucose, PCr and ATP, and an increase in lactate (Lowry et al., 1964; Brown et al., 1974). Results indicate that glycolytic flux may increase 7-fold in adult animals due to activation (possibly by increases in ADP, AMP and P_i) of phosphofructokinase, hexokinase and glycogen phosphorylase (Lowry et al., 1964; Bachelard et al., 1974).

Other metabolic changes due to ischaemia are:

(a) The release of intracellular K^+ (Bourke et al., 1978).

(b) A shift of NaCl and H_2O from the extracellular to the intracellular compartment (Hossmann et al., 1977).

(c) Changes in cyclic nucleotides coinciding temporally with the loss of cortical activity (Kobayashi et al., 1977).

(d) Disturbed monoamine metabolism (Brown et al., 1974). 24 hr after ligation of gerbil carotid artery, there was a 46% fall in DA on the

infarcted side with no change in NA concentration (Bowen & Davison, 1976).

(e) Altered glucose uptake. At 15 and 30 min post-embolization (injection into internal carotid artery of plastic 35μm diameter microspheres) there is a massive fall in [^{14}C]-deoxyglucose uptake in both deep and cortical structures. After 4 hr, a small zone of increased uptake persisted around each embolized microsphere (Schwartzman, 1978).

(f) Mitochondria from ischaemic brains shown impaired ATP formation and O_2 uptake (Kuwashima et al., 1978) and a fall in the respiratory control index due principally to reduced State 3 respiration (Rehncrona et al., 1979).

(g) Decreased capacity for lipid synthesis (Kosow et al., 1966).

(h) Decrease in membrane-bound glucose-6-phosphatase activity with a concomitant increase in the cytosol, possibly by activation of membrane-bound phospholipases (Rossowska & Dabrowiecki, 1978).

(i) Increased concentration of cerebral free fatty acids (FFA). During ischaemia, the labile pool of FFA rapidly increases in size, particularly during the first 3-5 min (Bazán & Cummings, 1969; Bazán, 1970; Bazán et al., 1971). This rapid production of FFA during the early phase of ischaemia is chiefly enzymic in nature and should be distinguished from post-mortem autolytic degradation of lipids (Banschbach & Geison, 1974). Bazán & Cummings (1969) suggested that the accumulation of FFA after decapitation was possibly due to depression of the energy dependent reacylation process. The ratio of total saturated to unsaturated FFA also suggest the involvement of phospholipase A_2 activity, known to be present in rat brain (Gallai-Hatchard & Thompson, 1965). The individual fatty acids showing the largest changes are arachidonic and stearic acids, followed by palmitic and oleic acids (Aveldaño & Bazán, 1975).

Kuwashima et al. (1978) have suggested that cerebral energy failure in ischaemic brain is related to the accumulation of FFA. Since lipid synthesis is depressed in ischaemic brain (Kosow et al., 1966; Kuwashima et al., 1976) it is possible that the greater part of the increased arachidonic and oleic acids are derived from mitochondrial membranes (Kuwashima et al., 1978), certainly not from myelin lipids (Bazán, 1970).

(j) Alteration in prostaglandin (PG) synthesis. Although Bosisio et al. (1976) demonstrated increases in PGE_2 and $PGF_{2\alpha}$ in brain cortex following 5 min ischaemia, levels of $PGF_{2\alpha}$ and its metabolite (13,14-dihydro-15-keto-$PGF_{2\alpha}$) were found to be unchanged during 2 hr ischaemia by Gaudet & Levine (1979) using the ischaemic gerbil model.

10. MORPHOLOGICAL CHANGES ASSOCIATED WITH ANOXIA, HYPOXIA AND ISCHAEMIA

Experimental animal studies indicate that different forms of severe O_2 deprivation, e.g. anoxia, hypoxia or ischaemia, all have one common neuropathological common denominator termed ischaemic cell change (Brierley et al., 1973).

Ischaemic cell change is a process that transforms a neurone to a more or less naked and very shrunken nucleus which will be engulfed by a phagocyte. The various stages involved in this process are:

(a) Microvacuolation of the cytoplasm which is considered as the earliest neuronal alteration resulting from anoxic-ischaemia (McGee-Russell et al., 1970).

(b) Swelling of mitochondria - observed after only 3 min of O_2 and glucose withdrawal from an isolated retina (Webster & Ames, 1965). With anoxia alone, 10 min are required before mitochondria appear swollen.

(c) Loss of extracellular space - causes membranes between the cellular elements to appear with tight junctions and accounts for the asphyxial

impedance increase due to a transport of H_2O and Cl^- into cellular elements (Van Harreveld & Malhotra, 1967; Hossmann et al., 1977). The release of intracellular K^+ into the extracellular compartment of the brain during hypoxia and ischaemia is related to the swelling of cerebral grey matter (Bourke et al., 1978).

(d) Swelling of endoplasmic reticulum and Golgi changes (Hossmann & Sato, 1970; Brierley et al., 1973).

(e) Loss and clumping of synaptic vesicles. Webster & Ames (1965) reported that the synapses between rod and bipolar cells of isolated retina were partially depleted of synaptic vesicles after 3 min of O_2 and glucose lack. An altered pattern of distribution of synaptic vesicles was observed after 3-4 min cerebral ischaemia in cats (Williams & Grossman, 1970). Clumping of vesicles in the region away from the synaptic cleft was seen in approx. 10% of synaptic endings and more than 2-fold increase in the number of presynaptic profiles devoid of vesicles.

These changes at the synapse may be one of the structural bases for the rapid disruption of cortical activity produced by brief periods of ischaemia and may provide further support for the idea that neurotransmission is more sensitive to O_2 lack than is axonal conduction.

(f) Swelling of astrocytes (Brierley et al., 1973).

11. RECOVERY AFTER HYPOXIC/ISCHAEMIC INSULT

11.1. Restoration of energy state and function

After a 10 min period of profound hypoxia (anoxia), the AEC shows full recovery from 0.39 to 0.92 within 15 min after O_2 restoration (Drewes & Gilboe, 1973b). The degree of recovery depends to a large extent on the duration of anoxia e.g. recovery of AEC is normal after 10 min anoxia but does not fully recover after 30 min anoxia (Drewes et al., 1973).

Using the isolated, perfused canine brain, Drewes & Gilboe (1973a) found that although the AEC showed almost complete normalization after 5 min reoxygenation, ATP took at least 30 min to recover in concentration. Levy & Duffy (1977) found a persistent decline in cortical ATP (77% of control at 24h) which would be expected to alter the kinetics of ATP-dependent reactions and therefore energy metabolism would still be abnormal even though the AEC returned to normal (99% of control at 24h). This idea, that recovery of the AEC does not necessarily imply functional recovery, is confirmed by Brown et al. (1974). These authors found that although recirculation following ischaemia rapidly restored PCr and allowed rephosphorylation of the adenine nucleotide pool, there was a persisting disturbance in monoamine metabolism, 30 min after 7½ min ischaemia, despite a restored energy state. This may reflect the requirement of ATP in the granular storage of monoamines.

Pre-exposure to anoxia significantly increases the survival time on re-exposure (Dahl & Balfour, 1964). This has been shown to be due not to increased reserves of ATP but more likely to effects on pyruvate. The increased lactate built up during the first exposure is metabolized to pyruvate during the return to air due to the consequent increased rate of aerobic glycolysis. If the animal is subjected to

repeated anoxia before the pyruvate has been dissipated, then one sees greater oxidation of NADH by the accumulated pyruvate, and ATP falls more slowly.

Using the Levine preparation which allows a direct comparison between the effects of hypoxia and ischaemia, (one carotid artery is clamped and the rat is then subjected to varying degrees of hypoxia - the unclamped hemisphere serves as a control) one finds that even partial or temporary interference with the blood supply to the cerebral hemisphere greatly adds to the threat of neurochemical damage in hypoxia and substantially interferes with the chemical recovery of the tissue (Salford et al., 1973a). After 30 min reoxygenation, neuronal abnormalities are only observed on the clamped side (Salford et al., 1973b).

Despite the fact that the energy state recovers and active pumping resumes to restore the ionic state (Hossmann et al., 1977), there are further complications to the restoration of function. Anoxia is known to induce vascular permeability changes causing subsequent cerebral oedema - a feature that often plays a major role in producing clinical effects (Schneck, 1971). After 1½ hr ischaemia in the baboon, re-perfusion is associated with an increase rather than a decrease in ischaemic oedema (Symon, 1978). Restoration of blood flow after a significant period of ischaemia may thus compound the problem of brain swelling since the post-hypoxic hyperaemia and brain swelling may lead to an increase of the intracranial pressure to such a degree that the cerebral circulation is severely curtailed (Freeman & Ingvar, 1968).

11.2. Membrane effects

An important point must be raised here and this is the distinction between complete and incomplete ischaemia. Although both

hypoxia with relative or incomplete ischaemia of 30 min duration may give rise to a permanent metabolic lesion and to irreversible histopathologic changes, animals can tolerate 30 min complete ischaemia without showing signs of such a lesion (Siesjö et al., 1977). CBF measurements indicate that recirculation occurs upon termination of the hypoxic-ischaemia and that the deterioration occurs later. There are several possible explanations for these observations which will be discussed below and in the following Section, 11.3.

A continued supply of O_2 may allow the O_2-dependent autolytic degradation of cell structure, possibly by the release of free radicals by peroxidation of membrane lipids. The involvement of free radicals in brain damage following ischaemic episodes has been suggested from observations that barbiturate (a free radical scavenger) anaesthesia helps protect the area of infarction in monkeys (Smith, 1977). When brain tissues were incubated in vitro in the presence of O_2 and suitable free radical initiators, appreciable amounts of lipid peroxides were formed (Westerberg et al., 1979). Selective changes in brain tissue phospholipids and FFAs occurred during peroxidation in vitro: decreased ethanolamine phosphoglyceride, no change in choline phosphoglyceride and decreases in arachidonic and docosahexaenoic acids. The presence of thiopental during incubation completely prevented the change in FFA composition (Smith et al., 1979).

Abnormal uncontrolled free radical reactions have been implicated in the initial molecular events for a variety of pathological processes (Demopoulos, 1973). Destruction of tissue results in the disruption of the normal spatial arrangements. This will lead to uncontrolled free radical production, particularly in the lipid bilayer structures of the cytoplasmic membranes.

One important factor in the prevention of peroxidative damage of brain tissues is the high ascorbic acid content of brain (Schaefer et al., 1975). Ascorbic acid is capable of initiating lipid peroxidation but the physiological level of ascorbic acid in the brain corresponds to a concentration at which it acts as a protective compound only. Catecholamines, phenothiazines and 5-HT have also been shown to be powerful inhibitors of lipid peroxidation in mitochondria and microsomes from rat liver (Schaefer et al., 1975).

Christophersen (1966) suggested that part of the oxidation of reduced glutathione (GSH) in homogenates was coupled to a mechanism that counteracts the peroxidation of membrane lipids. GSH can either act as a free radical scavenger, reduce disulphides by thiol-disulphide exchange mechanisms, or act as a substrate for glutathione peroxidase thereby quenching hydroperoxides formed as a result of peroxidative mechanisms. These reactions lead to the conversion of reduced (GSH) to oxidized glutathione (GSSG) with GSSG metabolized by glutathione reductase to GSH. Evidence for decreased GSH and increased GSSG would lend indirect support to the assumption that a reduction of brain tissue P_{O_2} may enhance formation of free radicals and induce lipid peroxidation. However during hypoxia there was neither a decrease in GSH nor an increase in GSSG concentrations in cortical tissue (GSH, 2μmole/g) or cisternal CSF (Folbergrová et al., 1979). This lack of change in GSH and GSSG concentrations could be due to high activity in the glutathione reductase reaction and a high capacity in the phosphogluconate pathway to generate NADPH for GSSG reduction.

Another important consequence for the release of lipid peroxides in hypoxia or ischaemia is the observation that the concentration of cellular peroxides (the cellular peroxide tone) can regulate prostaglandin synthesis (Hemler et al., 1979; Lands, 1979). Fatty acid oxygenation catalyzed by cyclooxygenase is the initial rate-limiting step in the conversion

of FFA to prostaglandins, thromboxanes and prostacyclin. Inhibition of cyclooxygenase by glutathione peroxidase provided evidence that cyclooxygenase catalysis required some peroxide which was normally generated by itself (Hemler et al., 1974).

Since the cellular peroxide tone regulates cyclooxygenase production of prostaglandins, thromboxanes and prostacyclin, it is important here to consider the action of these biologically-active compounds in relation to ischaemia. All primary prostaglandins and prostacyclin are formed in cerebral vessels - but not thromboxane A_2 (Wolfe & Coceani, 1979). Thromboxane A_2-like activity generated during human platelet aggregation by arachidonic acid causes contractions of the human basilar artery (Boullin et al., 1979). Thromboxane A_2 formed in damaged brain tissue and in aggregating platelets is considered a prime determinant of the vasospasm complicating thromboembolism and subarachnoid haemorrhage (Wolfe & Coceani, 1979).

Prostacylin can cause relaxation of cerebral arteries with the effect on the baboon common carotid artery being much more prolonged than on the intracranial arteries (Boullin et al., 1979). The outcome of an ischaemic assault will therefore depend to a large extent on the relative activities of the prostaglandins, thromboxane A_2 and prostacyclin within the brain.

Returning then to the influence of barbiturate anaesthesia on recovery from ischaemia. Perhaps anaesthetics act by suppressing neuronal excitability and thus reducing the metabolic demand for O_2. Levy & Duffy (1977) found supranormal PCr levels in gerbil brain recovering from ischaemia and suggested that this additional energy was being used to overcome cerebral oedema, inhibited protein synthesis and depleted DA. These increased energy demands, superimposed on the

energy required for recovering neurological function, might be responsible for the post-ischaemic hypermetabolism. Measures, such as anaesthesia, which would suppress neurological function, might channel the maximal available energy toward repair of cellular damage.

11.3. Mitochondrial function

As already described, one of the first ultrastructural changes observed in severe O_2 lack is mitochondrial swelling (Brierley et al., 1973). Mitochondria isolated from ischaemic brain show impaired ATP formation and reduced RCI due principally to a fall in State 3 respiration (Kuwashima et al., 1978; Rehncrona et al., 1979). Here also one finds that although recirculation after complete ischaemia gives mitochondria showing extensive functional recovery, mitochondria isolated after incomplete ischaemia show continued deterioration. Full recovery of mitochondrial function however does not necessarily imply full functional recovery. Schutz et al. (1973) observed that whilst mitochondrial function was only slightly impaired after 30 min total ischaemia, the animals (rabbits) failed to recover function after only 5 min ischaemia. It may be that changes take place in discrete regions of the brain only, which will be masked by measurements of whole brain mitochondria. Alternatively alterations in constituents of the cell other than the mitochondria could be responsible for the irreversible brain damage following brief periods of total ischaemia, e.g. nerve terminals.

Lazarewicz et al. (1972) suggested that the FFAs liberated in the brain during ischaemia could, by their inhibitory activity on energy metabolism of mitochondria, become one of the factors in the development of irreversible brain damage in ischaemia.

The brain is highly enriched in polyunsaturated fatty acids and since these fatty acids are largely esterified with the membrane

phosphoglycerides, only trace amounts of polyunsaturated fatty acids are normally present within the brain in the free form (Rowe, 1964). In fact Bruni et al. (1979) have found that injection of lysophosphatidylserine into mice decreased brain energy metabolism although both lysophosphatidycholine and lysophosphatidylethanolamine were without effect.

In 1955, Pressman & Lardy provided evidence that FFAs were the active agents from microsomes responsible for stimulating the respiration and latent ATPase of rat liver mitochondria. The uncoupling activity of FFAs may be distinguished from that brought about by agents breaking up mitochondrial structure in that it is readily reversible by the subsequent addition of bovine serum albumin. Borst et al. (1962) found that unsaturated long-chain FFAs were more effective than saturated FFAs in stimulating latent ATPase and identified oleic acid as the principal uncoupling agent.

A relationship between fatty acid structure and activity exists for inhibition of mitochondrial oxidation, stimulation of mitochondrial ATPase activity, and alteration of surface tension (Bjorntorp et al., 1964). This relationship suggests that the fatty acids may exert their inhibition of mitochondrial oxidation through changes in surface tension. According to this hypothesis, higher concentrations of fatty acids might be expected to damage the mitochondrial membrane sufficiently to allow leakage of respiratory cofactors. Both Bjorntorp et al. (1964) and Lochner et al. (1976) have found that replacing these cofactors, e.g. NAD^+, coenzyme A and cytochrome c does restore oxidation.

The uncoupling activity of fatty acids has however received more attention (Lazarewicz et al., 1972; Kuwashima et al., 1976). Some of the important properties of fatty acids are outlined below

with regard to liver mitochondria:

Are good respiratory substrates; inhibit liver mitochondria by uncoupling oxidative phosphorylation; inhibit oxidative processes; adenine nucleotide translocation and ATP-P_i exchange; influence ATPase activity; produce swelling; damage mitochondrial membranes; inhibit glycolysis and protein synthesis.

Addition of unsaturated fatty acids (oleic and arachidonic acids) impaired oxidative phosphorylation by rat brain mitochondria at a fatty acid concentration of 10^{-5}M (Kuwashima et al., 1976). The participation of FFAs in the development of metabolic disorders in ischaemic brain mitochondria has been supported by the following observations: 5 min post decapitative ischaemia produced (i) inhibition of mitochondrial respiratory activity, State 3, (ii) oxidative phosphorylation uncoupling, (iii) decreased RCI and the ADP/O ratio, (iv) 2-fold increase in the FFA content of these mitochondria (Lazarewicz et al., 1972). Similar changes were observed in in vitro anoxic mitochondria with no accumulation of FFA in oxygenated medium (Markelonis & Garbus, 1975).

Using mouse liver mitochondria, Chefurka (1966) suggested that phospholipase A was responsible for the accumulation of long-chain FFAs from phospholipids - a conclusion also reached by Bazán (1970) to account for the accumulation of cerebral FFAs after ischaemia. Although phospholipase A can facilitate loss of energy-linked functions such as respiratory control (liver mitochondria), the expression of its activity is not essential for the loss of energy-linked functions (Parce et al., 1978). It is likely that the reversal of damage and restoration of the RCI can only occur in the absence of measurable phospholipase A activity. Once expressed though, it contributes to the irreversible loss in mitochondrial metabolic functions by hydrolyzing

membrane phospholipids.

Phospholipase A activity has been identified in both human (Gallai-Hatchard et al., 1962) and rat brain (Gallai-Hatchard & Thompson, 1965). Phospholipase A activity can be differentiated into A_1 and A_2 forms in human and rat brains with slightly different pH optima - in rat these are 4.2 for A_1 and 5.5 for the A_2 form (Cooper & Webster, 1970). The activity of phospholipase A_2 is less than A_1 in all brain regions and neither enzyme requires Ca^{2+}. Of interest is the observation that the distribution of phospholipases A_1 and A_2 in subcellular fractions of rat brain was apparently quite different in two well-defined regions, e.g. cerebral cortex and hypothalamus (Bazán, 1969).

With regard to mitochondrial function, low concentrations (0.01-0.1mM) of FFA produce an increase in specific hexokinase activity in the supernatant fraction (solubilizing effect) without discernible damage to the rat brain mitochondrial membrane structure (Dománska-Janik et al., 1978). In comparison, hexokinase associated with the synaptosomal fraction was insensitive to the FFA concentrations used.

Treatment of mitochondria with phospholipase A_2 and C inhibits Ca^{2+} accumulation by 50% (Porcellati et al., 1978). Whether or not FFAs are causative in Ca^{2+} alterations is uncertain since there are two possible sequences of events:

(i) FFA release → Ca^{2+} alterations + energy imbalance → Ca^{2+} redistribution, or

(ii) Energy disturbances → Ca^{2+} changes → FFA release.

Finally, the release of FFAs and in particular arachidonic acid by phospholipase A_2 may be an important step in determining the extent of recovery due to regulation of the synthesis of prostaglandins, thromboxanes and prostacyclin (see also Section 11.2.).

In relation to lipid peroxidation it is important to note here that the structural integrity of mitochondria protects the membrane phospholipids from auto-oxidation by preventing the interaction between substrates and soluble pro-oxidant factors. Sharma (1977) demonstrated that phospholipids were substrates for lipid peroxidation in rat brain although neutral lipids were not. Choline phosphoglyceride was the most active and it is perhaps relevant to recall that choline phosphoglyceride constitutes the major phospholipid fraction in both mitochondria and synaptosomes (Eichberg et al., 1964).

11.4. Selective vulnerability

When survival times (in terms of EEG) of different brain regions were measured in an ischaemic cat preparation, they were found to vary by a factor of 4 (Sugar & Gerard, 1938).

Region	Survival time (sec)
Cerebellar grey	10-12 (time for disappearance
Cerebral cortex, grey	14-15 of potentials)
Subcortical white	20-22
Medulla	30-40

Further experiments have also indicated a heterogeneous response of the brain to incomplete ischaemia (Welsh et al., 1977). Here however, subcortical white matter was severely affected by an insult which caused only rather limited changes in the cortex and basal ganglia.

The caudate nucleus, despite its prediliction to metabolic derangement during ischaemia, appears capable of more prompt and complete metabolic recovery during recirculation than does the overlying cerebral cortex (Ginsberg et al., 1977). However when one considers the effect of hypoxia alone, similar metabolic and circulatory changes have been observed in rat cerebral cortex,

cerebellum and brainstem (MacMillan et al., 1974). These results suggest that there is no evidence for selective vulnerability - at least in the forms of hypoxia uncomplicated by ischaemia. This is in contrast to the work of Brierley et al. (1973) who indicated that neuronal damage (ischaemic cell change) is found in regions which exhibit selective vulnerability to hypoxic stresses including ischaemia. These regions are:

(i) Cerebral cortex, layers 3, 5 and 6.

(ii) Hippocampus, Sommer sector and endfolium.

(iii) Amygdaloid N, central and basolateral portions.

(iv) Cerebellum, Purkinje and basket cells.

(v) Brain stem, certain sensory nuclei in infants and young children, occasionally in adults.

The spinal cord is the most resistant while involvement of the thalamus, striatum, pallidum and subthalamic nucleus is variable.

If the brain does exhibit selective vulnerability then this will ultimately affect the outcome of an hypoxic-ischaemic insult.

11.5. The "no reflow phenomenon"

As already described, complete cerebral ischaemia of duration greater than 7 min leads to swelling of the vascular endothelial and perivascular glial cells (Hossman & Sato, 1970). This may result in the obstruction of the vessel's lumen and in a serious impairment of the blood recirculation following ischaemia, i.e. the "no reflow phenomenon". Many of the experiments already cited have indicated the requirement of an adequate perfusion pressure in both maintaining the AEC during hypoxia and in promoting full recovery. The "no reflow phenomenon" in rabbits affects the brain stem, basal ganglia and thalamus earlier and to a greater extent than the cortex with the degree of severity being related to the duration of ischaemia

(Fisher, 1973). After 15 min ischaemia there was a 90% area of no reflow. Possible mechanisms are:

(i) Vascular obstruction due to swelling of endothelial and perivascular glial cells as a result of energy failure.

(ii) Increased blood viscosity due to fibrin clots, platelet thrombi or "sludging" (Cuypers & Matakas, 1974).

(iii) Constriction of vascular lumen - impaired CBF can be prevented by intravenous infusion of NA (Hossmann & Sato, 1970).

It should be pointed out here that the "no reflow phenomenon" has not been consistently observed and may in fact reflect inadequate reperfusion pressures - this however only serves to enhance its possible significance clinically.

11.6. Re-growth of damaged neurones

Lesions in the CNS can be brought about by a variety of causes, e.g. experimentally-induced chemical or surgical lesions, and perhaps more relevantly, ischaemia-induced areas of infarcted tissue within the brain as a possible consequence of a stroke.

Repair and re-growth of damaged neurones show quite distinct differences depending on the source. In CNS (septal neurophil) the degenerating axon terminals remained in contact with their postsynaptic sites for up to one month following the lesion. On contrast, in the peripheral nervous system (superior cervical ganglion) the degenerating terminals were all removed by 24 hr after operation (Raisman, 1977).

The formation of new synaptic connections in the peripheral nervous system involves:

(i) Ability of neurones to produce growing axon sprouts.

(ii) Elongation of axon sprouts through the tissues and along the pathways lead to the "target" tissues.

(iii) Cessation of elongation and the formation of terminal specializations which innervate the postsynaptic structures.

In the CNS however, cut fibres (lesion of fimbria to hippocampo-septal region) do bud but do not elongate towards the target cells (Raisman, 1978). Reinnervation can occur though by sprouting of nerve terminals (un-cut) from the contra lateral side. Perhaps here it is the glial cells that prevent the axon sprouts from crossing the lesion and reaching their original postsynaptic targets.

12. BRAIN DYSFUNCTION

12.1. Introduction

In clinical medicine, anoxia and ischaemia rank near the top as common causes of brain injury under circumstances that affect every period of life (Siesjö & Plum, 1973). It is probable that lack of O_2 underlies many serious physiological and chemical derangements in the brain.

As already described, total withdrawal of O_2 supply to the brain, e.g. circulatory arrest, will lead to first evidence of brain dysfunction, complete loss of consciousness, within approximately 10 sec (Symon, 1978). The actual anoxic damage to each neurone may very well be similar in a wide variety of circumstances, but the total clinical and neuropathological picture will depend very much upon the character of the initial incident and upon the secondary or sequential changes which ensue. Studies on comatose patients have indicated that the clinical outcome cannot be predicted (Cold, 1978).

The factors influencing recovery from anoxia and ischaemia have been described (see Section 11) and therefore this section will consider other factors influencing cerebral O_2 utilization including in particular a discussion of disorders of the ageing nervous system.

12.2. Ageing of the brain

A study of human ageing has shown that even in normal elderly subjects free from any signs of vascular disorder, there was a 23% reduction in glucose consumption of the whole brain (Birren et al., 1963) although the mean value for CMR_{O_2} for men at 71 was not significantly different from the rate for young adults (Lassen, 1959).

Total cerebral metabolism has been found to remain remarkably constant in most physiological conditions. O_2 consumption by cerebral grey matter is greater than by white matter and since the cerebral cortex forms most of the grey matter, it may be assumed that a subnormal CMR_{O_2} signifies a hypometabolism of the cerebral cortex. This has been confirmed and two different types of hypofunction have been found:

(i) Acute cerebral depression with a reduction in the level of consciousness, e.g. semicoma and coma

(ii) Chronic cerebral degenerative diseases with a reduction of intellectual faculties, e.g. organic dementia.

Point (i) will be considered here although it does not necessarily fall into the class of ageing brains. Coma, as well as being produced by anoxia and ischaemia, can be elicited by several other metabolic alterations.

A. Hypoglycaemia

A simple lack of glucose has been recognised for many years as causing changes in behaviour and in EEG although P_{O_2} and P_{CO_2} in the bloodstream are normal (Bachelard, 1976). The dependence on glucose as the main respiratory substrate of the brain has been well documented (McIlwain & Bachelard, 1971) and currently the concept that ketone bodies can be used routinely by the normal brain as a respiratory fuel is being reconsidered (Hawkins & Biebuyck, 1979). Ketone bodies may be

considered supplementary to glucose, partially supplying specific areas of brain but incapable of supporting the energy requirement of all regions.

Deprivation of glucose can be tolerated to a greater degree that O_2 lack provided that oxygenation is maintained (Schneck, 1971). Hypoglycaemia induces slow wave and polyspike convulsive activity in the EEG without changes in the energy state of the cerebral cortex (Lewis et al., 1974). Energy failure is therefore not responsible for the initial depression in cerebral function nor the convulsive activity although prolonged convulsive activity will lead to energy failure, coma and isoelectricity.

B. Hyperammonaemia

Chronic liver disease can often result in gross neurological and psychiatric disturbances, with convulsions leading to coma and death. The principal cellular abnormalities are seen in brain astrocytes (Bowen & Davison, 1976). These are mainly in the form of glial hypertrophy with glutamine increased 4-fold although glutamate and GABA remain unchanged.

The formation of glutamine is the major cerebral mechanism for removing ammonia (McIlwain & Bachelard, 1971) and the accumulated glutamine is partly converted to α-oxoglutaramate, a metabolite not normally found in appreciable amounts. Whether α-oxoglutaramate is a key toxic metabolite in the clinical condition has yet to be determined.

Hyperammonaemia is also accompanied by reduced CMR_{O_2} and increased glycolysis (Bachelard, 1976).

C. Ageing

In the course of normal ageing, the brain loses 5 to 12% of its

weight and up to 50% of cortical neurones (Terry, 1979). The morphological changes also include the progressive appearance of neuritic plaques (areas of amyloid surrounded by axonal endings filled with degenerating mitochondria and lysosomes); of neurofibrillary tangles (intraneuronal paired helical filaments of an abnormal protein); of granuolar vacuolation in various brain cells; and of lipofuscin (an "ageing pigment" found in increasing concentrations in the cerebellum, neocortex, midbrain, hippocampus and medulla). Not all of the changes occur in all ageing brains (Tomlinson, 1979).

Using *in vivo* non-invasive dual wavelength spectrophotometry, cytochrome a, a_3 was found to be 30% reduced under "resting" conditions in both the mature and aged rat brain (Sylvia & Rosenthal, 1978). There were no significant age-related differences although the presence of lung pathology markedly affected the redox level of cytochrome a,a_3. These *in vivo* experiments would indicate that dysfunction of the mitochondrial respiratory chain is not a direct or primary consequence of chronological ageing. This does however contrast with *in vitro* experiments described by Chefurka (1966). Aged mitochondria *in vitro* demonstrated reduced respiratory and latent ATPase activity which were found to be due to the release of FFA from phospholipids. Other ageing lesions such as swelling, loss of nucleotides and Mg^{2+} were found to be unrelated to the inactivation of the DNP-stimulated ATPase activity in aged mitochondria.

The volume of brain occupied by extracellular space is reduced to about half that characteristic of normal animals (anoxia and ischaemia both reduce extracellular space, Van Harreveld & Malhotra, 1967; Hossmann *et al.*, 1977) and therefore a decreased capacity to transport ions and small molecules might be anticipated in the brains of senescent animals (Bondareff, 1973).

Ageing is characterized principally by a general decline of tolerance to adapt to forced metabolism rather than by a disturbed cerebral blood flow. This view is supported by the observation that there were significant decreases in acetylcholinesterase, succinate dehydrogenase and cAMP-activated protein kinase activity in an age-dependent manner in both rat and ox brains (Meier-Ruge et al., 1976). Benzi et al. (1979) have also noticed an age-dependent decline in lactate dehydrogenase, citrate synthase, malate dehydrogenase, total NADH-cytochrome c reductase and cytochrome a,a_3 activities in rat brain (14-60 weeks of age).

Finally there is the observation that the response in aged rats to denervation in the septal area is quantitatively less pronounced although qualitatively similar to that of young adult rats (Scheff et al., 1978). This reduction in reactive growth may reflect a decreased capacity of the aged brain to remodel its circuitry and restore lost function. The reason for this diminished plasticity is unknown - a reduced vascular supply is however not totally responsible.

Several theories have been proposed to account for the ageing process and these are summarized as follows: cross-linking, especially DNA (Zs-Nagy, 1978); free radicals (Barber & Bernheim, 1967); radiation; rate of energy expenditure; accumulation of waste products and altered endocrine function.

12.3. Disorders of the ageing brain

During the last few decades increasing attention has been given to the study of the diseases of old age and, in general, to the various problems confronting old people. This applies particularly to the countries of the West, where demographic, economic and social developments have already brought about a striking structural change

in the population. The declining mortality and the enhanced possibilities of preventing and curing diseases mean that hereditary characters in general and genetically-conditioned diseases occurring in the older age groups in particular will acquire increased importance.

The development of a mental illness which starts in later life is commonly related to progressive cerebral changes, commonly the result of a degenerative process, the two main categories of which are the "vascular" (loosely covered by the term 'cerebral arteriosclerosis'), which affects the large and small cerebral vessels, and the "senile", which involves the nervous tissue more directly, and includes cerebral atrophy, the formation of senile plaques and the degeneration and loss of nerve cells. This latter category will be considered in greater detail.

A. Arteriosclerosis

When one considers the adequacy of O_2 delivery to the brain and the regulation of CBF in patients with arteriosclerosis, one must question the assumption that the gradual atheromatous narrowing of the larger cerebral arteries is actually a common cause of progressive presenile and senile chronic brain disease. Lassen (1959) warns here that the possibility of primary parenchymatous processes must be considered in all patients showing chronic progressive brain disease regardless of circumstantial evidence of arteriosclerosis.

B. Dementia

In the western world, elderly patients account for nearly half the total mental hospital population and perhaps at least a third of these patients have senile dementia making this the commonest organic nervous disease in mature adults (Bowen & Davison, 1976).

There are a wide variety of neurological diseases presenting with dementia; cerebral atrophies (presenile, senile and multi-infarct dementia), Parkinson's disease, Huntington's Chorea, chronic inflammatory diseases, metabolic disorders, dialysis dementia (aluminium toxicity), hydrocephalus and tumours. It is interesting that older patients with brain tumours will present as "demented" whereas younger patients will show the typical signs of for example, headaches (Blessed, 1980).

The principal dementias to be considered here are those associated with predominantly diffuse cortical lesions: senile dementia, senile dementia of the Alzheimer's type (SDAT), and multi-infarct dementia.

Comparison of the morphological changes observed in brains from patients suffering senile dementia with normal, age-matched controls shows that there are no statistically-significant differences between the two groups in, for example, loss of brain weight and distribution of neuritic plaques and neurofibrillary tangles. However SDAT does show more profound neuropathological changes with gross atrophy of the cerebral cortical sulci, granulo-vacuolar degeneration in hippocampal pyramidal cells, and the presence of plaques, neurofibrillary tangles and argyrophilic inclusion bodies (Reisine et al., 1978). There is a strong correlation between the concentration of cortical plaques and impaired intellectual functioning (dementia) and although one can have plaques without senile dementia, one does not find senile dementia without plaques (Tomlinson, 1980).

Neurochemically the most significant alteration is a marked decrease in choline acetyltransferase (CAT) activity, particularly in the hippocampus (Perry et al., 1977). The vulnerability of the hippocampal cholinergic system in old age and dementia may be associated

with memory derangement. An important point to be made here is the selective vulnerability of the hippocampus to hypoxic stress (Brierley et al., 1973) as described in Section 11.4. CAT activity also decreased in the caudate nucleus, amygdala, and cerebral cortex, suggesting a selective loss of cholinergic cells in these brain areas (Reisine et al., 1978).

Despite all the information gained on the consequences of the dementia syndrome, its etiology is still speculative. In patients whose mental condition is completely dominated by severe loss of intelligence, the cerebral O_2 uptake is reduced to about 2.0ml/100g/min (60-70% of normal), while in those with less apparent dementia the value lies between 2.5-3.0 (Lassen, 1959). Severe organic dementia is associated with the same low values as CMR_{O_2} as were found in coma. These two conditions are however, clinically strikingly different. Subjects with such degrees of dementia have no loss of consciousness. Possibly the gradual but complete loss of many cortical cells does not affect cortical function as profoundly as the severe hypofunction of all cortical cells found in coma. Lassen has further demonstrated that in SDAT and multi-infarct dementia, CBF is decreased in proportion to the decrease in CMR_{O_2} and that these decreases occurring in the left hemisphere correlated better with dementia than any right hemisphere deficit (Lassen, 1979).

This brings out another important point: that the degree of decline in interneuronal communication during senescence may well prove to correlate with declining intellectual function. Under normal circumstances, an external stimulus can be shown, by computed axial tomography, to have increased CBF and CMR_{O_2} in discrete regions of the brain, always accompanied by activation in the frontal cortex where integration takes place (Lassen, 1979). However, in many demented

patients the frontal lobe fails to respond although, for example, a visual stimulus will have increased CBF and CMR_{O_2} in the visual cortex. It is an interesting possibility that the dementia syndrome is a manifestation of defective interneuronal communication specifically in relation to the higher integration centres. We have already seen that synaptic transmission is more vulnerable to O_2 lack than is axonal conduction (see Section 10.) and that alterations in cholinergic function in dementia occur in regions of the brain considered to be especially vulnerable to hypoxia. Reports on receptor binding studies are inconclusive at the present time. Changes have been found in other neurotransmitter systems in senile dementia although these too have not been confirmed either way. For example, Smith & Swash (1979) found that GABA synthesis was spared in SDAT in contrast to the conclusions of Reisine et al. (1978). The complex interactions between different neurotransmitter systems will make precise conclusions elusive here.

Whether or not reduced CMR_{O_2} is a consequence or a causative factor in dementia is therefore uncertain. Hypoxia alone seems unlikely to be involved in senile dementia since dementia performance cannot be reversed by hyperbaric O_2 (Lassen, 1979). In contrast however, ischaemia is obviously involved in multi-infarct dementia where recovery after an ischaemic insult should be maximized possibly by the maintenance of CBF and the use of barbiturate anaesthesia (see Section 11).

Accumulation of lipofuscin in the hippocampus is thought to be almost diagnostic of senile dementia (Bowen & Davison, 1976). Since there is no evidence of cytotoxicity of lipofuscin, it may be that lipofuscin acts in a protective manner as a sink for free radicals (Terry, 1979). As already described in Section 11 this would prove advantageous because as lipid peroxidation is autocatalytic, once started it can compound the injuries initiated by other factors and thus

contribute to the overall pathology.

Various other factors have been implicated in the etiology of the dementias such as the involvement of a transmissible agent (e.g. in Creutzfeldt-Jakob disease and Scrapie in animals) or a mutant protein of neurotubulin as the cause of the tangles. The possibility of a genetic determinant was discussed by Larsson et al. (1963) and further supported by Roth (1979) on the observation that senile dementia seems to be characterized by "threshold" effects in a similar manner as the genetically determined diabetes mellitus.

In a large scale Swedish survey, Larsson et al. (1963) determined that the morbidity risk for senile dementia among the parents and children of those affected could be assessed at 4.3 times the corresponding morbidity risk in the general population. The implication of a hereditary factor here does not exclude the possibility that senile dementia is sometimes conditioned by exogenous factors. Some of the conclusions drawn from this survey were:

(i) Clear clinical differences at advanced stages between senile dementia and SDAT, and between senile dementia and senility, which - in spite of the patho-anatomical similarities observed - give evidence that senile dementia and SDAT are two different disease entities.

(ii) There are no indications that environmental factors of a socio-medical nature play a part in the onset of senile dementia.

(iii) Absence of any increased morbidity risk for other psychoses among the relatives.

(iv) Although genetically determined, senile dementia is not the expression of a monohybrid recessive gene since the variability with regard to both the age of onset and the rate of progression of the main clinical symptoms are not compatible with this conclusion.

13. AIM OF RESEARCH PROJECT

Appreciable changes can occur in local cerebral P_{O_2} without concomitant changes in ATP production, even at mild degrees of hypoxia at which signs of changed cerebral functioning are first evident (Duffy et al., 1972; MacMillan & Siesjö, 1972; Bachelard et al., 1974). The changes in EEG which occur in hypoxia indicate an effect on synaptic function, a suggestion which is supported by Dolivo (1974) who demonstrated that synaptic transmission was more vulnerable to O_2 and glucose deprivation than was axonal conduction.

Much of the work already done on hypoxia - and particularly the more dramatic conditions of anoxia and ischaemia - has involved experiments in whole brain which might well mask possible biochemical changes occurring in discrete regions or organelles. Therefore in this project, isolated nerve-endings (synaptosomes) have been used with the hope of pinpointing any biochemical abnormalities occurring in various degrees of O_2 deprivation to some aspect of synaptic transmission. Synaptosomes have been shown to have a full complement of glycolytic and respiratory enzymes and they retain the permeability characteristics of the intact nerve terminals (Bradford, 1975).

The aim has been to characterize the process of synaptosomal O_2 consumption and demonstrate its possible dependency on various factors such as the composition of the medium with regard especially to any selective dependency on exogenous respiratory substrates. The experiments involve the use of a Rank oxygen electrode to study the rate of synaptosomal O_2 uptake over short time periods in contrast to previous experiments utilizing Warburg manometers to monitor O_2 consumption for time periods greater than 30 min (Bradford, 1969).

It may be possible to demonstrate the existence of a critical O_2 concentration where there is a change in the kinetics of the O_2 consumption process. From this one can establish conditions resembling hypoxia *in vivo* and proceed to study the metabolism of synaptosomes in hypoxic conditions. Adaptation of the oxygen electrode should allow incubations to be made at defined constant O_2 concentrations using three conditions most likely to show up any alterations; normoxia, hypoxia and anoxia.

All aspects of the synaptic transmission process can be studied using the synaptosome preparation ranging from general metabolism to neurotransmitter synthesis and release. Those aspects already established from whole animal experiments and which can be studied more directly here are for example:

(1) Direct utilization of O_2 by the synaptosome for mitochondrial oxidative phosphorylation and the influence of the synaptosomal limiting membrane on the function of the intrasynaptosomal mitochondria in maintaining the "adenylate energy charge".

(2) The possible breakdown of synaptosomal membranes and the influence of any accumulation of FFAs on synaptosomal O_2 consumption.

(3) The influence of reduced P_{O_2} on O_2-dependent enzymes with lower affinities for O_2 than the mitochondrial respiratory enzymes. In particular, with regard to the behavioural abnormalities observed in hypoxia, one could study the activities of tyrosine and tryptophan hydroxylases. It has been estimated that $< 0.1\%$ of O_2 consumed by the brain is utilized in the direct synthesis and metabolism of NA, DA and 5-HT (Davis & Carlsson, 1973b) and therefore any reduction in O_2 supply to the brain is likely to affect these reactions given the relatively high K_m values of some of their enzymes for O_2.

14. MODELS USED TO STUDY ANOXIA, HYPOXIA AND ISCHAEMIA

14.1. Introduction

There are many different approaches to the study of anoxia, hypoxia and ischaemia - each method alone provides limited information that can then be pieced together to produce a more complete picture. A wide range of laboratory animal species have been used in the hope of relating the information thus obtained to events in man. However interpreting data from various species is limited by the possibility of species differences - not least to their survival during O_2 deprivation. For example, using an apparently similar experimental design, the dog recovers good neurological function after 25 min whereas the rat is severely crippled after 5 min total cerebral ischaemia (Schutz et al., 1973). Similarly exposure to 5.6% O_2 for 1 hr will produce sleepy rats in contrast to unconsciousness in man (Davis & Carlsson, 1973b). This is due to a difference in the position of the oxyhaemoglobin dissociation curve: rat P_{50} = 42mmHg and man P_{50} = 29mmHg. There is even evidence of strain differences where Sprague-Dawley rats show a relatively low mortality rate in hypoxia (Kinnula et al., 1978). Finally differences in lipid composition have been found between squirrel monkeys (Saimuri sciureus) and rats (Sun & Sun, 1972). For example in monkeys the ratio of phosphatidylethanolamine to phosphatidylcholine is higher, in myelin there is more docosahexaenoic acid and in synaptosomes there is more oleic acid and less arachidonic acid.

14.2. Whole animal experiments

The most commonly used method for studying the response of the whole animal to hypoxia and anoxia is to place the animal in a chamber which is flushed through with gas of a known composition. After a set time interval, the animal is killed and the brain is rapidly removed for analysis. This method has many advantages in that the functional

interactions of the complete organism are retained. The biggest disadvantage though is the possible masking of biochemical alterations which may only have occurred in discrete regions. Methods for fixing the brain *in situ* are improving so that at least fairly small brain regions can now be dissected.

Alternatively anaesthetized animals can be used where continuous measurement of blood gases and pH can be monitored whilst altering the O_2 content of the inspired gas. Here one must take into account the anaesthetic used and make certain that the blood pressure and body temperature are maintained since these can all affect the response of the animal to hypoxia (Siesjö, 1978).

As for studying ischaemia, the ideal experimental animal is the Mongolian gerbil. In most animals when the carotid arteries are ligated, redistribution of blood to the brain can occur via the circle of Willis (Bowen & Davison, 1976). The Mongolian gerbil lacks the circle of Willis and therefore complete cerebral ischaemia can be produced by clamping the carotid arteries. Other methods of producing ischaemia include compression ischaemia (infusion of artificial CSF into the cisterna magna), decapitation, cross clamping of the aorta, inflation of a pressure cuff around the neck, and unilateral carotid ligation followed by N_2 exposure (to distinguish ischaemia from anoxia).

14.3. Isolated cerebral tissues

The following methods all involve the use of a physiological medium gassed with low or zero O_2 to provide the hypoxic or anoxic conditions for the isolated cerebral tissues. The most sophisticated of the methods is probably the isolated canine brain preparation (Drewes *et al.*, 1973) although this would seem to have few advantages over the intact, anaesthetized animal experiments of Siesjö's laboratory.

Finally there is the use of subcellular fractions. Here one can study the function of individual components free from interactions with other cellular components - this is not necessarily an advantage but can in itself provide a lot of information. The complex nature of the brain and the well established selective vulnerability of various brain regions to hypoxia and ischaemia suggest that there are further discrete structures such as nerve terminals that could be specifically affected by hypoxia and ischaemia but in which changes would be masked by measurement in the brain as a whole. Synaptosomes (isolated nerve-endings) have been selected for this study because it seems to be the process of synaptic transmission that is particularly vulnerable to O_2 lack (Dolivo, 1974) and the functional changes observed in hypoxia are more probably due to a failure in transmission rather than a failure in energy production (Duffy et al., 1972; MacMillan & Siesjö, 1972; Bachelard et al., 1974). The same advantages and disadvantages apply to the use of subcellular fractions as with cell suspensions but even more attention must be paid to recognizing the purity and reproducibility of the particular preparation.

15. SYNAPTOSOMES - THE EXPERIMENTAL TOOL

15.1. Introduction

When brain tissue is homogenised in iso-osmotic sucrose solutions under conditions of moderate shear force, the nerve terminals are torn away from their axons and reseal to form nerve-ending particles (Gray & Whittaker, 1962; De Robertis et al., 1962a). Isolated nerve-ending particles were originally termed "Synaptosomes" by Whittaker et al. (1964) and have been shown to be well organised cytoplasmic units retaining much of the biochemistry as well as the morphological compexity of the original pre-synaptic nerve ending. For reviews see Marchbanks & Whittaker, 1969; Bradford, 1975; Jones, 1975, and McIlwain, 1975.

15.2. Preparation

Although the cerebral cortex from a wide range of mammals has been the principal source for the preparation of synaptosomes, they may also be prepared from other defined regions of the CNS, e.g. spinal cord (Osborne et al., 1973).

The conditions originally described by Gray & Whittaker (1962) (suspension medium 0.32M sucrose; tissue concentration 10% w/v; use of a smooth-walled Perspex and glass homogenizer with pestle mechanically driven at 840 rpm; clearance between pestle and mortar of 0.25mm at 30mm diameter) appear to be close to optimum for mammalian brain samples, particularly when considering cholinergic nerve-endings (Whittaker & Dowe, 1965) which make up 15% of cortical synapses (McLennan, 1965). Judging by the distribution of markers, cells are at least 85% destroyed and nerve terminals are converted to synaptosomes with not less than 70% and possible close to 90% efficiency (Marchbanks & Whittaker, 1969).

Separation of the constituents of the homogenate is achieved by an initial low speed centrifugation which separates whole cells, nuclei and tissue debris. The supernatant fraction is centrifuged to sediment the crude mitochondrial fraction which is then further subfractionated on a discontinuous sucrose density gradient. Ficoll, a sucrose polymer, is sometimes used in preference to sucrose because of its low osmotic pressure in solution (Kurokawa et al., 1965). Morpho-biochemical characteristics were preserved when synaptosomes were isolated in a Ficoll medium which was found to be non-inhibitory to oxidative phosphorylation (Abdel-Latif, 1966). However Morgan et al.(1971) found that rat brain synaptosomes isolated on isotonic Ficoll-sucrose density gradients were highly sensitive to osmotic shock in comparison to those isolated on sucrose density gradients.

15.3. Morphology

Electron microscopy of synaptosomes shows that a large number of nerve-endings have continuous membrane profiles which enclose synaptic vesicles, complex vesicles, vacuoles and one or more mitochondria (Jones & Bradford, 1971). Often one finds present attached post-synaptic thickenings. Taking these observations together, Jones & Brearley (1972) concluded that synaptosomes accurately reproduce the major ultrastructural features of synaptic junctions. Synaptosomal diameter is 0.5-1.0μm (Bradford, 1972) and synaptosomal volume has been estimated as 3.88μl (Marchbanks & Campbell, 1976).

15.4. Metabolic properties

When interpreting metabolic data from synaptosomal studies, one must bear in mind that although synaptosomes make up a large proportion of the "synaptosome fraction" there are two other important components. These are the contaminating myelin fragments and free mitochondria. Contamination by myelin fragments will particularly

affect estimates of respiration rates since they will contribute protein to the "synaptosome fraction" but they will be devoid of respiratory activity thus producing an underestimate of the true value.

Similarly because mitochondria show higher specific activities regarding the oxidation of substrates such as glutamate when compared to synaptosomes, then even a few per cent contamination by free mitochondria will contribute a disproportionate increase in O_2 uptake on for example, the addition of ADP.

A. The presence of an intact synaptosomal membrane

During homogenization procedures the presynaptic nerve terminals together with the postsynaptic thickenings are torn away from the postsynaptic membranes and either "bud off" from or break away from the axons. There is considerable evidence that any torn membrane very quickly reseals to form an apparently complete surface over the synaptosome (Jones & Bradford, 1971).

The osmotic properties of synaptosomes, as shown by their ability to shrink and swell in media of different tonicity, is evidence for a continuous limiting membrane (Marchbanks, 1967; Keen & White, 1970). Further, synaptosomes retain soluble cytoplasmic components such as lactate dehydrogenase (Johnson & Whittaker, 1963), diffusable ions such as K^+ (Marchbanks, 1967), pyridine nucleotides (Lindall & Franz, 1967), and putative neurotransmitters.

When synaptosomes are incubated in physiological salines they behave like intact cells, showing high linear respiration, producing lactate and amino acids, generating PCr and ATP and accumulating K^+ against a concentration gradient (Ling & Abdel-Latif, 1968; Bradford, 1969; Bradford & Thomas, 1969). This property of accumulating K^+ against a concentration gradient - not found in mitochondria or frag-

mented membranes - suggests the existence of a K^+ diffusion potential, possibly equivalent to the resting membrane potential (Blaustein & Goldring, 1975). De Belleroche & Bradford (1973) in fact estimated the membrane potential as -27mV.

B. Oxygen consumption

Reports on synaptosomal O_2 consumption give values in the region of 60μmoles O_2/hr/100mg protein in the presence of at least 5mM glucose (Bradford, 1969; Balfour & Gilbert, 1970). The endogenous respiration of synaptosomes diminishes rapidly after approximately 20 min and does not support either K^+, ATP or PCr accumulation which is in contrast to the effect of exogenous glucose at 10mM which stimulates respiration 3-fold (Bradford, 1969). Longer-term experiments have indicated that suspensions of synaptosomes incubated with glucose in Warburg respirometer vessels show linear respiration for periods of 3-4 hr despite early losses of K^+ and amino acids (Bradford et al., 1975).

Respiration rates are determined to a large extent by the composition of the incubation medium. Verity (1972) found a large stimulation of synaptosomal respiration due to Na^+ and interpreted this as being primarily due to an increased generation of ADP by the Na^+, K^+-ATPase system. Extrusion of Na^+ elevates ADP levels which in turn stimulates respiration. The presence of a Na^+, K^+-ATPase system in the plasma membrane of the synaptosome (Bradford et al., 1966; Ling & Abdel-Latif, 1968) has also been implicated in the Na^+-induced stimulation of glucose oxidation (Diamond & Fishman, 1973).

When synaptosomes are incubated in Krebs-Ringer-tris containing high Na^+ (124mM) and low K^+ (6.2mM), they respire well in a linear fashion with glucose and at a substantially higher rate with pyruvate as substrate. In contrast, when the medium contained high K^+

(180mM) but no Na^+, synaptosomal respiration failed to respond to glucose (Bradford, 1969). Rates of respiration are generally higher in a Na^+ medium (Booth & Clark, 1978).

Electrical pulses and raised potassium levels were found to increase both respiration and glycolysis (Bradford, 1970). The response to raised K^+ is not reproducible as significant increases in O_2 consumption were not always found (Balfour & Gilbert, 1970; Osborne et al., 1976).

The Ca^{2+} concentration also influences the rate of respiration of synaptosomes. The absence of Ca^{2+} greatly accelerated respiratory (30%) and glycolytic rates (Bradford et al., 1973). Decreasing the Ca^{2+} concentration of the medium to low levels is well known to stimulate respiration and glycolysis in slices (McIlwain, 1952) even though the presence of Ca^{2+} in concentrations of 1-2mM is required to maintain long-term respiration (Dickens & Greville, 1935). Lazarewicz et al.(1978) have suggested that Ca^{2+} preferentially inhibits anaerobic glycolysis and Dodd et al.(1971) concluded that Ca^{2+} may limit glucose uptake. Ca^{2+} stabilizes plasma membranes (Baker, 1972) and therefore its omission is likely to increase the permeability of the synaptosomal plasma membrane to cations, for example, Na^+ which has been demonstrated to increase respiration (Verity, 1972).

In the absence of glucose, K^+ concentration in synaptosomes falls to half the value found during glucose-supported respiration and there are similar falls in ATP and PCr (Bradford et al., 1975). Glutamate (10mM) is capable of stimulating a high unstable respiratory rate which becomes linear on the addition of glucose and which after 1 hr of incubation gives values for ATP and PCr only half those found with glucose (Bradford, 1969).

Since glutamate (±glucose) either prevented synthesis or caused 50-80% loss of synaptosomal ATP and PCr, it may be acting by depolarising the synaptosomal membrane causing Na^+ influx and consequent ATP hydrolysis with the release of ADP to stimulate respiration (Bradford, 1969). Also in the absence of glucose, the expected general accumulation of amino acids in the medium did not occur (Bradford et al., 1975). Pools of glutamate and GABA in tissue were substantially diminished in size suggesting the use of endogenous amino acids as respiratory substrates since synaptosomes show considerable O_2 uptake in the absence of added substrate and are able to oxidise both added glutamate and GABA.

It is likely that the main source of glutamate for use as an endogenous respiratory substrate by synaptosomes is from its conversion by glutaminase from glutamine. Glutaminase has been localised in nerve-endings (Salganicoff & De Robertis, 1965) but their glutamine content is very low (Bradford & Thomas, 1969) in agreement with their low content of glutamine synthetase (Salganicoff & De Robertis, 1965). The most likely source of glutamine in nerve-endings is from the CSF and from the conversion of glutamate to glutamine by glutamine synthetase localised in both glia and the neuronal perikarya (Weiler et al., 1979). Although glutamine's contribution to total glutamate, aspartate and GABA in nerve-endings is substantial relative to glucose (50-70% after 45 min), its contribution to energy metabolism under normal conditions seems to be minimal (Bradford et al., 1978).

C. Neurotransmission

Synaptosomes have been shown to possess many of the enzymes necessary for the synthesis and metabolism of neurotransmitters (see Bradford, 1975), and both electrical pulses and raised K^+ levels are capable of releasing putative neurotransmitters (Bradford, 1970). For

example, ACh is released from incubated, metabolically-maintained synaptosomes of cerebral cortex by both raised K^+ levels and electrical pulses. This release is Ca^{2+}-dependent and results in the synthesis of additional ACh by the synaptosomes (Haga, 1971; De Belleroche & Bradford, 1972). Ca^{2+} entry - enhanced by increasing internal Na^+ - is essential for the release of neurotransmitters both *in situ* and *in vitro*. Treatment with K^+ induces a pattern of Ca^{2+} entry that closely follows the theoretical pattern of depolarization suggesting a causal relationship between the two events (Blaustein & Wiseman, 1970). Spontaneous neurotransmitter release from synaptosomes can be augmented when the external Ca^{2+} concentration is zero by interfering with mitochondrial sequestration of Ca^{2+} (Silbergeld, 1977). Elevated K^+, in the absence of Ca^{2+}, can release GABA from newly-synthesized pools in preference to endogenous pools whereas the Ca^{2+}-dependent GABA efflux, facilitated by K^+, was similar for both pools of GABA (Haycock *et al.*, 1978).

D. Membrane structure

The lipid composition of synaptosomes was found to be similar to that of the whole brain homogenate but they can be distinguished from myelin by their low cerebroside content and from mitochondria by their lack of cardiolipin (Eichberg *et al.*, 1964).

More than 80% of the main lipid classes in the whole homogenate can be recovered in the primary and secondary cell fractions indicating that no appreciable autolysis had occurred and that there is no loss through the manipulative procedures for the preparation of synaptosomes (Eichberg *et al.*, 1964).

The nerve-ending membrane has a chemical composition that distinguishes it from plasma membranes of non-neuronal cells, including:

(i) high lipid/protein ratio

(ii) high percentage of carbohydrate

(iii) high levels of membrane-bound enzymes

(iv) diverse receptors for neurotransmitters and drugs

(v) a unique complement of polypeptides (Smith & Loh, 1979).

Striking similarities in chemical and functional composition make it clear that the nerve-ending membrane is in almost all respects simply an extension of the somal plasma membrane.

Synaptosomes are active in the metabolism of phosphoglyceride acyl groups, possibly regulated by a deacylation-reacylation phenomenon. There was rapid incorporation of radioactive palmitic, oleic and arachidonic acids into the crude mitochondrial fraction from guinea-pig cerebral cortex after only 7.5 min *in vitro* incubation (Baker *et al.*, 1976). Sun & Su (1979) found that synaptosomes incorporated radioactive arachidonic acid into phosphatidylinositol within 1 hr after intracerebral injection into mouse brain. Baker *et al.* (1976) have indicated a relatively high rate of synthesis *de novo* of phosphatidylinositol at the nerve-ending when compared with the whole brain and this may underline the possible functional importance of phosphatidylinositol at the synapse.

Phospholipases A_1 and A_2 are both present in the synaptosome (Bazán, 1969) along with a lysophospholipase (Kunze, 1973). The deacylation of phosphatidylethanolamine has been shown to be increased by ACh, NA and 5-HT possibly by stimulating phospholipases A_1 and A_2. Similarly, N^6, O^2-dibutyryl cyclic AMP decreased the deacylation of phosphatidylethanolamine, possibly by inhibition of phospholipase A_2 (Kunze, 1973). Synaptosomes contain 4.5mg of unesterified fatty acid per g protein (Price & Rowe, 1972). After incubation for 90 min with 1mM 5-HT, NA or adrenaline, there was a significant increase in

unesterified fatty acids in the synaptosomal suspension with NA showing a selective increase in palmitic acid when compared to stearic and oleic acids (Price & Rowe, 1972). NA and adrenaline preferentially increased the release of unesterified fatty acid into the medium. It is possible that these changes induced by putative neurotransmitter agents reflect changes in structure and therefore properties of the synaptic membrane during synaptic transmission.

The high polyunsaturated acyl group content of the synaptosomal membranes has been related to the functional involvement of the membrane in active transport, since an alteration of these polyunsaturated acyl groups may inevitably inhibit the activity of the transport Na^+, K^+-ATPase (Sun & Sun, 1972). The phospholipids of brain and retina - two excitable tissues - are characterized by the presence of the polyunsaturated fatty acid docosahexaenoic acid, (C22:6), (Breckenridge et al., 1971). Although the functional role of a high docosahexaenoic acid content in brain membranes has not been fully elucidated, its presence is seemingly important in providing the molecular conformation and hydrophobic character of the membrane. Importantly, a membrane containing a high level of polyunsaturated acyl groups is also highly susceptible to breakdown by peroxidase and lipoxygenase.

16. MEASUREMENT OF P_{O_2} USING OXYGEN ELECTRODES

The oxygen electrode is now the most commonly used instrument for O_2 determinations. Although the use of any oxygen electrode is frequently termed the "polarographic method", the original polarographic design is seldom used (Lessler, 1972). Most of the oxygen electrodes used today are based on the Clark electrode design which involves placing the cathode and anode under a thin, O_2-permeable membrane. Essentially the Clark oxygen electrode consists of a platinum disk electrode separated from a calomel half-cell by a very thin film of potassium chloride solution, the platinum electrode being 0.6-0.8 volt negative with respect to the calomel half-cell. The film of KCl solution is separated from the reaction mixture by a thin polyethylene or teflon membrane, which is freely permeable to O_2. The current which flows through this electrode is directly proportional to the O_2 activity and the electrode is attached to a recorder so that direct traces of the electrode current can be obtained.

The oxygen electrode measurements depend on the electrolysis of O_2 at a weakly negative cathode. The electrode is therefore polarized by a constant voltage supply and it produces a current proportional to the number of O_2 atoms reduced at the cathode. It is important to remember that in common with most electrode systems, the Clark oxygen electrode measures the activity and not the concentration of O_2 present. This has been shown to be the case by determining the response of the electrode in KCl solutions of different molarities, each of which had been thoroughly equilibrated with air at 25°C. The current obtained when the electrode was in 1M-KCl was 99% of that when it was in H_2O, despite the fact that the concentration of O_2 in 1M-KCl is only 73% of that of air-saturated H_2O (Chappell, 1964).

The activity coefficient of O_2 in solution, γ, may be calculated:

$$\frac{\log \gamma}{I} = K$$

where I = ionic strength and K = a constant which depends to some extent on the nature of the electrolyte. Calculation of the effective ionic strength is not easy for complex mixtures and the presence of non-electrolytes will affect the solubility of O_2. For experiments using a Ringer-type medium, one can use values for O_2 solubility as determined by Umbreit et al.(1972) taking care to remember that O_2 solubility is temperature sensitive.

Several methods exist for determining the solubility of O_2 in a complex medium. The most commonly used method is that of Chappell (1964) where the electrode is calibrated directly by measuring the oxidation of a known amount of NADH by a sub-mitochondrial particle preparation. The reaction involved is

$$NADH + H^+ + \tfrac{1}{2} O_2 \rightarrow NAD^+ + H_2O$$

and because the NADH can be determined spectrophotometrically, the corresponding O_2 concentration can be determined. A similar method replacing the mitochondrial preparation by the chemical N-methyl-phenazonium methosulphate (PMS) has been described by Robinson & Cooper (1970) and a further chemical method using ascorbic acid oxidase has been detailed by Capietti et al.(1977). Dixon & Kleppe (1965) have also described a simple method for correction of the O_2 solubility factor due to the presence of various dissolved substances.

The Clark oxygen electrode was adapted by Chappell (1964) to be used in conjunction with a water-jacketed reaction vessel for the measurement of mitochondrial respiration. An extension of this design is a piece of apparatus which is capable of measuring both respiratory rates and the redox state of cytochrome c simultaneously

in isolated cellular suspensions (Wilson et al., 1977). Further adaptation allows the measurement of respiration in perfused isolated small organs using a "respirometer" (Turner et al., 1968). The use of a membrane-covered electrode in all these systems does however mean that a sensitivity limit significantly lower than 0.1μM cannot be achieved (Degn, 1977).

Although the Clark oxygen electrode in a closed system is ideal for measuring fast respiration rates, it has several disadvantages when one wishes to determine the dependency of respiratory rates on P_{O_2}. For example using this system, the P_{O_2} passes through the non-linear range (final part of the trace) in such a short time that it is not justified to assume that the results are steady-state rates of respiration as functions of P_{O_2} (Degn & Wohlrab, 1971). Similarly this technique, as well as for example manometers or bacterial luminescence, requires that the biological system under investigation is responsible for the major fraction of the O_2 consumption and/or that the response of the biological system to changed P_{O_2} is rapid relative to the rate of change of P_{O_2} (Jones & Mason, 1978).

Therefore in order to study the O_2 dependence of various O_2-utilizing systems, various pieces of apparatus have been designed to allow the continual introduction of O_2 in order to maintain a constant P_{O_2} or a constant P_{O_2} long enough to achieve new steady state conditions. The first example is that of Garrison & Ford (1970) called an oxystat. This was useful for tissue membranes, e.g. the chorioallantoic membrane of the embryonic chick, but was not suitable for cell suspensions. About this same time Degn (1969) developed a piece of apparatus for the simultaneous measurement of steady state values of respiration rate and the oxidation level of respiratory pigments at low P_{O_2}. In this system called a "respirograph" a stirred suspension of, for example, mitochondria

is exposed to a gas phase, the oxygen tension of which is increasing linearly with time. This technique rests upon the assumption that when a steady state exists, i.e., when the O_2 concentration in the liquid (mitochondrial suspension) is constant, the rate of O_2 consumption by the respiring sample and the rate of O_2 into the liquid are equal. Thus if the O_2 concentration in the gas is known, and the O_2 concentration in the liquid is measured polarographically, together with the appropriate constant, the rate of respiration can be calculated. This method has been used successfully by Clark et al.(1976) in determining the K_m for O_2 of rat brain mitochondrial respiration (0.1μM).

Further to this is the oxystat of Jones & Mason (1978) which is capable of rapidly providing and maintaining a constant P_{O_2} from 1-150mmHg in suspensions. The voltage output from a Clark oxygen electrode in an incubation vessel regulates O_2 flow into the vessel to control the solution P_{O_2} at a predetermined level. The advantage of this system is that it allows one to study systems in which intracellular function and control of oxidases respond only slowly to changes in P_{O_2}.

CHAPTER 2

METHODS AND RESULTS

Chapter 2. METHODS AND RESULTS

1. MATERIALS

1.1. Animals

CFHB Wistar-remote, derived from Anglia Animals.

Diet, Oxoid 41B. (University of Bath animal house).

CSE Wistar, specific pathogen free.

Diet, Spratts laboratory diet 1. (St. Thomas's Hospital Medical School animal house).

1.2. Chemicals

All general reagents were of AR grade when available. Fine and special chemicals used are listed below:

s-Acetylthiocholine iodide; 5,5'-Dithiobis (2-nitrobenzoic acid), Ellman's reagent; Dimethoxypropane; 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride, INT; Strophanthin-G, ouabain. From BDH Chemicals Ltd., Poole, Dorset.

Adenosine-5'-diphosphoric acid, disodium salt; Lactate dehydrogenase, EC 1.1.1.27, rabbit muscle; β-Nicotinamide-adenine dinucleotide, oxidized form, grade II; β-Nicotinamide-adenine dinucleotide, reduced form, grade II. From Boehringer Corporation (London) Ltd., Bell Lane, Lewes, East Sussex.

Ficoll. From Pharmacia (Great Britain) Ltd., Prince Regent Road, Hounslow, Middlesex.

Albumin, bovine, essentially fatty acid free <0.005%; Albumin, bovine, Fraction V; Arachidonic acid, methyl ester, grade I;

Docosahexaenoic acid, methyl ester, grade I; Heptadecanoic acid, methyl ester, grade I; N-methyl-N-nitroso-p-toluene sulfonamide; Oligomycin; Tetra isopropylpyrophosphoramide. From Sigma London Chemical Company Ltd., Fancy Road, Poole, Dorset.

1.3. Apparatus

Rank oxygen electrode (perspex). From Rank Brothers, High Street, Bottisham, Cambridge.

Centrifuges:

Method Heaton & Bachelard (1973), see Section 3.

M.S.E. High speed 18 (8 x 50ml) and M.S.E. Superspeed 50 (3 x 25ml swing-out and 10 x 10ml).

Methods 1-5.

M.S.E. High speed 18 (8 x 50ml), Beckman L5-65 Ultra-centrifuge (6 x 38ml swing-out), M.S.E. Superspeed 50 (10 x 10ml).

Method 6.

M.S.E. High speed 18 (8 x 50ml), M.S.E. Prepspin 50 (3 x 25ml swing-out and 10 x 10ml).

2. STATISTICS

Student's t-test, two-tailed, was used to test the degree of significance of results (Clark, 1969).

Standard Error of Mean (S.E.M.) = Standard Deviation (S.D.)/ $\sqrt{n}$

3. PREPARATION OF SYNAPTOSOMES

3.1. Method

Synaptosomes were prepared from the cerebral cortex of adult male rats, 200-300g body weight. The rats were killed by decapitation (between 9-11 am) and the brains rapidly removed onto filter paper

soaked in ice-cold 0.32M sucrose. After separating from the cerebellum, the cerebral cortex was scraped free of white matter. The cerebral cortex from one rat was then homogenized in 10 volumes of ice-cold 0.32M sucrose by six up-and-down strokes in 30 sec, followed by a 60 sec cooling period on ice and a further six up-and-down strokes in 30 sec. Total time, 2 min. The brain homogenate was then ready for fractionation as described below.

Due to several factors, including moving this project from Bath to London, there have been slight variations in the preparative procedure as summarized in Table 3.1. However, in the vast majority of cases, the preparation of synaptosomes has been based upon the method of Bradford et al. (1975). The exception to this was the use of the method described by Heaton & Bachelard (1973) in the initial stages of the project. The principle difference here is the long preparation time involved due to centrifugation of the density gradient at 53,000g x 2 hr (Heaton & Bachelard, 1973) compared to 75,000g x 1 hr (Bradford et al., 1975).

Electron micrographs of subcellular fractions obtained by both methods are shown in Section 4.6.1.

TABLE 3.1. Summary of variations in the preparation of synaptosomes

Method Number	Length of pestle (cm)	Clearance (mm)	Homogenization speed (RPM)	Number of washes of P_1
1	3.0	0.25	1200	2
2	3.0	0.25	840	2
3	3.0	0.25	840	None
4	3.0	0.50	840	None
5	1.3	0.25	840	None
6 (LONDON)	1.3	0.25	900	None

Figures 3.1.1. and 3.1.2. give full details of the method of preparation of synaptosomes and following is a key to the symbols used, (Gray & Whittaker, 1962).

Key to symbols:

H Homogenate

P_1 Nuclear pellet

P_2 Crude mitochondrial pellet

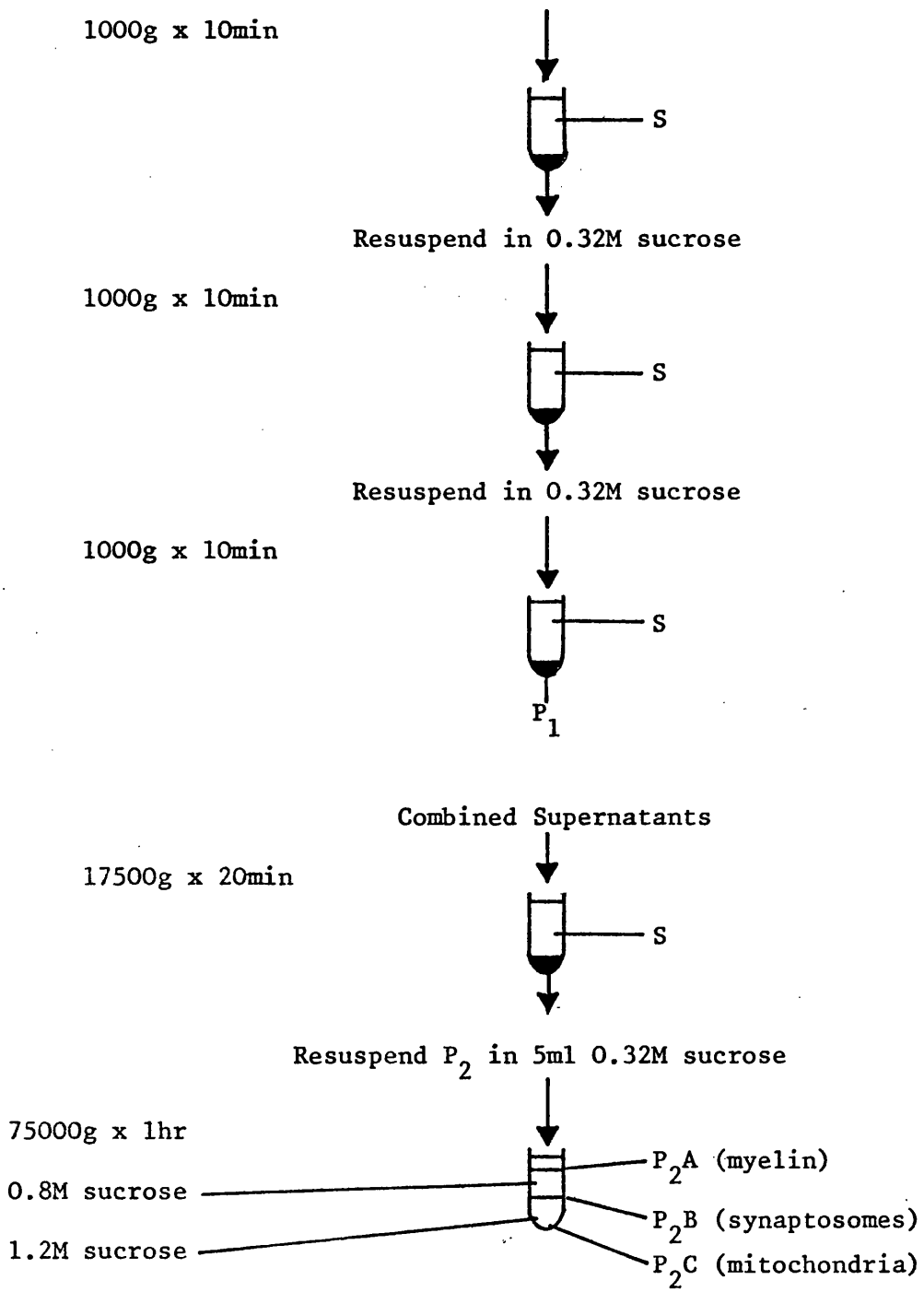
S Combined supernatants

P_2A Myelin)

P_2B Synaptosomes) Subfractions of P_2

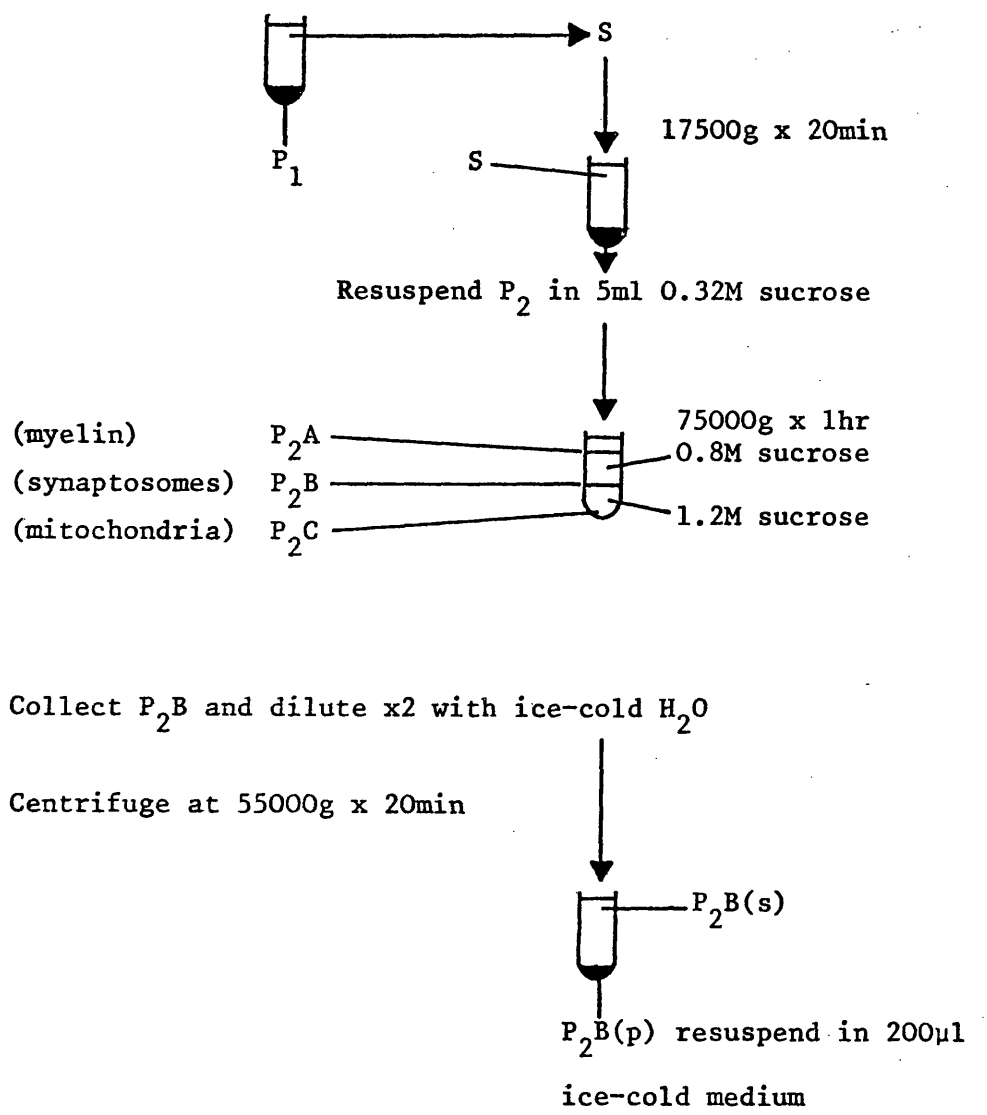
P_2C Mitochondria)

FIGURE 3.1.1. PREPARATION OF SYNAPTOSOMES BY THE METHOD OF BRADFORD et al. (1975)



All procedures at 0-4°C.

FIGURE 3.1.2. METHOD USED FOR THE PREPARATION OF SYNAPTOSOMES



All procedures at 0-4°C.

4. CHARACTERISATION OF FRACTIONS

Interpretation of biochemical and physiological experiments involving subcellular fractions depends on the purity of the fractions and this is determined by the initial homogenization procedure and the subsequent centrifugation scheme. Relatively small changes in homogenization conditions have been shown to alter the distribution of bound ACh with the yield of synaptosomes in the homogenate falling as the shear rate increases (Whittaker & Dowe, 1965). Since the rate separations in most cases are based on size (Cotman, 1972), the homogenization technique dramatically influences the resulting rate separation. With brain, recycling (washing) is not so satisfactory since fragile structures such as synaptosomes become damaged by resuspension and recentrifugation, leading to a decrease rather than increase in yield of the appropriate structure.

A combination of enzymic data and transmission electron microscopy is used to characterise the fractions obtained during the preparation of synaptosomes. The principle enzyme markers are: (1) Acetylcholinesterase (AChE), EC 3.1.1.7, which is associated with the synaptosome limiting membrane and other plasma membranes, (Rodriguez de Lores Arnaiz *et al.*, 1967); (2) Succinate dehydrogenase (SDH), EC 1.3.99.1, for mitochondria and (3) Lactate dehydrogenase (LDH), EC 1.1.1.27, as a cytoplasmic marker, (Johnson & Whittaker, 1963).

Methods for assaying these enzyme markers are given below, followed by the results and discussion of the characterisation of fractions obtained by the different methods used. An account of the application of electron microscopy is also given.

4.1. PROTEIN ASSAY

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

4.2. ACETYLCHOLINESTERASE ASSAY

Acetylcholinesterase activity was assayed according to the method of Ellman et al. (1961). Units of activity are μmoles substrate hydrolysed/min/ml.

Since 90% of the cholinesterase activity in rat brain is due to acetylcholinesterase (Aldridge & Johnson, 1959), the distribution of this enzyme is not appreciably different from that of the total cholinesterase. However, the use of acetylcholine and n-butyrylcholine to determine AChE and pseudocholinesterase in the various fractions revealed a marked difference in their distribution. Koelle (1955) suggested that pseudocholinesterase is present mainly in glial cells. The main substrate of pseudocholinesterase is butyrylcholine which is also probably hydrolyzed slowly by the AChE of most species.

To obtain an indication of the contribution made by pseudocholinesterase [BuChE], an inhibitor of BuChE was used and this rate was subtracted from one obtained in the absence of any inhibition, i.e. [AChE + BuChE] minus [BuChE] reflects [AChE]. (Alternatively an inhibitor of AChE could be used).

The inhibitor of BuChE used was 10^{-5}M tetraisopropyl-pyrophosphoramide (0.41mg/100ml) in 0.1M phosphate buffer, pH8. [A stock solution (0.041g/100ml) of tetraisopropylpyrophosphoramide does not survive freezing and thawing].

4.3. SUCCINATE DEHYDROGENASE ASSAY

The activity of succinate dehydrogenase was determined according to Porteus & Clark (1965). One unit of activity is equivalent to the oxidation of 1μmole of succinate in 15 min at 37°C.

4.4. LACTATE DEHYDROGENASE ASSAY

Lactate dehydrogenase activity was determined using the method of Kornberg (1955) with 0.15M Tris-HCl, pH 7.4, as the buffer. Units of activity are μmoles substrate oxidised/min/ml. Considerable free lactate dehydrogenase activity may be detected in the myelin fraction, presumably derived from soluble components of the resuspended crude mitochondria fraction. Although lactate dehydrogenase is generally recognised as a soluble enzyme, the conditions of low ionic strength used for subcellular fractionation allows non-specific binding of proteins to membrane fragments - a property exhibited by the most basic of the five lactate dehydrogenase iso-enzymes (Fonnum, 1967).

4.5. RESULTS AND DISCUSSION OF CHARACTERISATION STUDIES

The results presented in Tables 4.5.1. to 4.5.4. confirm those of Whittaker & Dowe (1965) by showing that slight variations in the preparative procedure can alter the distribution of the various marker enzymes and thus the degree of contamination of the recovered synaptosomes.

According to Barondes (1974), higher speeds of centrifugation and reduced washing to collect P_2 increases the yield of synaptosomes but at the same time increases the degree of contamination with other membranes. The principle contaminants of the synaptosome fraction are myelin fragments carried down from the gradient layer above and empty membrane sacs (0.2-1.0μm diameter) of unknown origin (Whittaker, 1968).

Comparison of the various methods shows:

(1) Decreasing the initial homogenization speed from 1200 to 840 rpm increases the relative specific activity of acetylcholinesterase and occluded lactate dehydrogenase in the synaptosome fraction with an accompanying fall in these activities in the myelin fraction. (The relative specific activity of an enzyme is determined as "Activity in

subfraction as % activity in parent fraction divided by protein in subfraction as % protein in parent fraction". A value greater than one indicates purification of a particular fraction).

(2) No washing of P_1 increases the relative specific activity of acetylcholinesterase and occluded lactate dehydrogenase in fraction P_2.

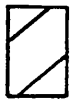
(3) The changes observed between Methods 5 and 6 do not simply represent the differences caused by the slight change in homogenization speed. Other factors here are (a) the calibration curves provided for the MSE High Speed 18 centrifuges (used to produce fraction P_2) were slightly different as brought out from the results in Table 4.5.1. The primary spin in Method 6 is probably closer to 2,000g than 1,000g used in all other Methods. (b) The dimensions of the density gradient were smaller in Method 6 and this may account for the less efficient separation of fractions P_2A and p_2B.

(4) The results shown in Table 4.5.2.(ii) suggest that the high R.S.A. values for AChE found in fraction P_2A are likely to be due to the presence of BuChE.

FIGURE 4.5. THE RELATIVE SPECIFIC ACTIVITIES OF MARKER ENZYMES IN SUBFRACTIONS OF P_2 (CRUDE MITOCHONDRIA PELLET)

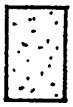
Values are Mean ± S.E.M. (n = 4) for subcellular fractionation according to Bradford et al. (1975) as described in Section 3, Method 5.

KEY



P_2A (myelin)

P_2B (synaptosomes)



P_2C (mitochondria)

SDH Succinate dehydrogenase

AChE Acetylcholinesterase

LDH Lactate dehydrogenase

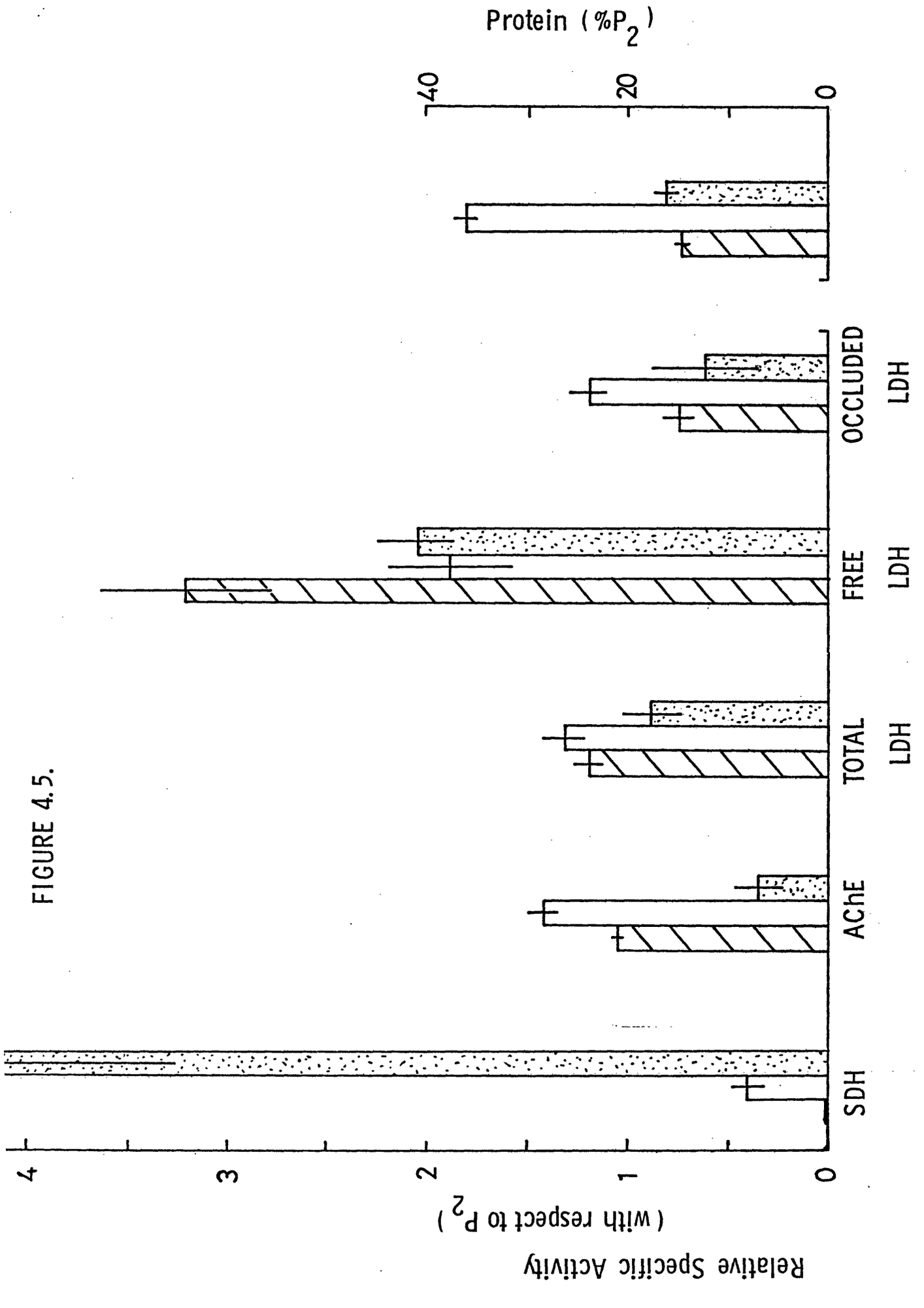

Protein (%P2)
40
20
0
SDH
ACHE
TOTAL
LDH
FREE
LDH
OCCLUDED
LDH
Relative Specific Activity
(with respect to P2)
4
3
2
1
0

FIGURE 4.5.

TABLE 4.5.1. Distribution of protein in rat brain subcellular fractions

	METHOD			
	1	2	5	6
	n = 4	n = 6	n = 4	n = 4
FRACTION				
	Protein as % Homogenate			
P_1	15.52(0.94)	18.18(1.91)	31.07(1.78)	49.71(2.00)
P_2	57.42(5.34)	47.05(3.26)	31.17(2.25)	25.05(2.92)
S	34.85(4.02)	30.26(3.25)	21.04(1.35)	23.40(2.16)
P_2A	12.48(1.57)	10.20(1.14)	4.60(0.59)	3.66(0.22)
P_2B	18.87(1.77)	12.43(0.63)	11.38(1.22)	9.70(0.68)
P_2C	9.51(0.25)	5.35(0.52)	5.00(0.12)	5.17(0.37)
Recovery	107.80(9.78)	91.48(7.68)	83.28(4.10)	98.16(5.82)
	Protein as % P_2			
P_2A	14.33(4.88)	22.75(4.08)	14.61(0.89)	14.96(1.34)
P_2B	33.40(2.09)	24.26(3.25)	36.28(1.46)	39.37(1.94)
P_2C	16.92(1.40)	11.29(0.66)	16.26(1.21)	20.97(1.04)

Values given are Mean (S.E.M.)

TABLE 4.5.2.(i). Distribution of acetylcholinesterase activity in rat brain subcellular fractions

	METHOD			
	1	2	5	6
	n = 2	n = 5	n = 4	n = 4
FRACTION				
	Relative Specific Activity in terms of H			
P_1	0.68(0.13)	0.59(0.03)	0.75(0.01)	0.97(0.05)
P_2	0.89(0.07)	1.00(0.03)	1.27(0.07)	1.68(0.19)
S	1.15(0.23)	0.89(0.06)	0.94(0.06)	0.80(0.13)
Recovery	95.97%	89.17%	82.18(3.40)%	108.08(9.64)%
	Relative Specific Activity in terms of P_2			
P_2A	3.09	0.93	1.06(0.03)	1.52(0.21)
P_2B	1.24	1.66	1.42(0.07)	1.08(0.07)
P_2C	0.34	1.35	0.35(0.03)	0.24(0.04)

Values given are Mean (S.E.M.). The activity of AChE described here includes a contribution from BuChE (see Section 4.2.).

TABLE 4.5.2.(ii). Distribution of acetylcholinesterase and butyryl-cholinesterase activity within subcellular fractions of rat brain

Fraction	AChE + BuChE	AChE	BuChE
	Relative Specific Activity in terms of H		
Method 1. P_1	0.68	0.68	0.71
n = 2 P_2	0.89	0.89	0.92
S	1.15	1.18	0.67
	Relative Specific Activity in terms of P_2		
P_2A	3.09	2.81	5.12
P_2B	1.24	1.33	0.56
P_2C	0.34	0.36	0.37
	Relative Specific Activity in terms of H		
Method 2. P_1	0.70	0.69	0.82
n = 2 P_2	1.12	1.27	0.65
S	0.93	1.13	0.06
	Relative Specific Activity in terms of P_2		
P_2A	0.95	0.97	0.82
P_2B	1.23	1.29	0.71
P_2C	0.26	0.28	0.15

TABLE 4.5.3. Distribution of succinate dehydrogenase activity in rat brain subcellular fractions

	METHOD		
	2	5	6
	n = 5	n = 4	n = 3
FRACTION			
	Relative Specific Activity in terms of H		
P_1	0.73(0.08)	0.80(0.05)	1.17(0.12)
P_2	1.41(0.11)	1.34(0.13)	0.98(0.33)
S	0.04(0.02)	0.03(0.02)	0.01(0.01)
Recovery	83.48%	67.01%	86.17(6.02)%
	Relative Specific Activity in terms of P_2		
P_2A	0.06	0.00	0.00
P_2B	0.41	0.41(0.11)	0.36(0.01)
P_2C	3.58	4.18(0.89)	3.15(0.49)

Values given are Mean (S.E.M.).

TABLE 4.5.4.(i). Distribution of total lactate dehydrogenase activity in rat brain subcellular fractions

	METHOD			
	1	2	5	6
	n = 2	n = 5	n = 4	n = 4
FRACTION	Relative Specific Activity in terms of H			
P_1	0.83(0.27)	0.84(0.12)	0.63(0.05)	0.76(0.10)
P_2	0.90(0.03)	0.76(0.04)	1.02(0.17)	0.73(0.13)
S	1.80(0.17)	0.92(0.09)	1.57(0.28)	1.53(0.10)
Recovery	121.07%	75.38%	91.84(4.33)%	90.42(5.29)%
	Relative Specific Activity in terms of P_2			
P_2A	2.38	0.72	1.19(0.07)	1.46(0.22)
P_2B	1.18	1.09	1.32(0.12)	1.23(0.15)
P_2C	0.55	0.76	0.88(0.16)	0.76(0.16)

Values given are Mean (S.E.M.). See also Tables 4.5.4.(ii) and (iii).

TABLE 4.5.4.(ii). Distribution of free lactate dehydrogenase activity in rat brain subcellular fractions

	METHOD			
	1	2	5	6
	n = 2	n = 5	n = 4	n = 4
FRACTION				
	Relative Specific Activity in terms of H			
P_1	0.71(0.12)	0.52(0.03)	0.54(0.09)	0.51(0.06)
P_2	0.69(0.04)	0.69(0.02)	0.29(0.01)	0.28(0.04)
S	1.77(0.04)	1.20(0.10)	3.67(0.61)	2.85(0.21)
	Relative Specific Activity in terms of P_2			
P_2A	2.37	3.95	3.20(0.42)	3.10(0.22)
P_2B	0.90	2.98	1.88(0.29)	1.41(0.15)
P_2C	0.43	5.03	2.05(0.47)	1.49(0.28)

Values given are Mean (S.E.M.). See also Tables 4.5.4.(i) and (iii).

TABLE 4.5.4.(iii). Distribution of occluded lactate dehydrogenase activity in rat brain subcellular fractions

	METHOD			
	1	2	5	6
	n = 2	n = 5	n = 4	n = 4
FRACTION				
	Relative Specific Activity in terms of H			
P_1	1.79(1.05)	0.58(0.10)	0.75(0.09)	0.99(0.17)
P_2	2.07(0.06)	0.88(0.14)	1.22(0.22)	1.10(0.16)
S	1.88(0.87)	0.38(0.14)	0.43(0.18)	0.43(0.02)
	Relative Specific Activity in terms of P_2			
P_2A	2.43	1.34	0.75(0.07)	1.11(0.20)
P_2B	1.68	3.20	1.21(0.09)	1.19(0.15)
P_2C	0.77	0.72	0.62(0.09)	0.60(0.13)

Values given are Mean (S.E.M.). See also Tables 4.5.4.(i) and (ii). Occluded LDH is calculated as the difference between total LDH and free LDH activity.

4.6. ELECTRON MICROSCOPY

The electron microscope is often used to assess the purity of subcellular fractions along with chemical and enzymic data. However, there are several important problems associated with the use of the electron microscope as an analytical tool and these will be discussed later, see Section 4.6.2.

4.6.1. Methods and Results

The first method to be described is a general scheme for preparing most biological tissue for transmission electron microscopic examination. The second method is a more rapid procedure which is more applicable for routine use (I. Duce, personal communication).

METHOD 1

Fixation

Add 3% glutaraldehyde (in 0.2M cacodylate buffer, pH 7.3 , containing sucrose (45mg/ml)) to pelleted tissue sample.

Fix at 0-4^{o}C for 3hr or overnight.

Wash with buffer, three times each for 30 min or until the smell of glutaraldehyde disappears.

Add 1.33% osmium tetroxide in buffer.

Fix at room temperature for 1hr (in fume cupboard).

Wash with buffer, two times each for 30 min.

Dehydration

Series of ethanol: 20%, 35%, 50%, 65%, 75%, 85%, 96%, 100%

Dehydration times: 10-15 min in 20%-75%

30 min in 85% and 96%

3 x 30 min in 100%

(Can be left overnight in 75%).

Add propylene oxide : ethanol (1:1, v/v) for 1hr, then 100% propylene oxide, two times each for 30 min.

Embedding

Place the prepared samples in plastic embedding capsules and add propylene oxide:Spurr low viscosity resin (1:1, v/v) overnight. The propylene oxide will evaporate to give 90-95% resin.

Add fresh resin for one day at room temperature.

Change to fresh resin again and polymerise at 60°C for three days or longer.

Alternative dehydration procedure

After fixation of the tissue, add dimethoxypropane (acidified by 1 drop of HCl in 50ml) for 5 min.

Wash with 100% ethanol.

METHOD 2

Fixation

Add 2½% glutaraldehyde (in phosphate buffer, pH 7.3, containing sucrose (45mg/ml)) to pelleted tissue sample.

Fix at 0-4°C for 3hr or overnight.

Wash with buffer, three times each for 30 min or until the smell of glutaraldehyde disappears.

Add 1% osmium tetroxide in buffer.

Fix at room temperature for 1hr (in fume cupboard).

Wash with distilled water, three times each for 30 min.

Dehydration

Series of ethanol: 30%, 50%, 70%, 90%, 100%.

Dehydration times: 10 min in 30%-90%

3 x 20 min in 100%

Embedding

Place prepared samples in plastic embedding capsules and add Spurr resin for 2hr to infiltrate.

Add fresh Spurr resin and polymerise at 60°C overnight or longer.

(When using Taab resin, first add resin:ethanol (1:1, v/v) for 2hr. Add neat resin and polymerise at 60°C for at least 48hr).

In both methods, the glutaraldehyde-fixed pellet is removed from its centrifuge tube and is cut up into small pieces before subsequent fixation in osmium tetroxide. The ideal size for optimum penetration of the fixative is a cube with $1mm^2$ faces - or less if possible.

Sectioning

Ultrathin sectionswere cut with a glass knife using a Reichert OM U3 Ultramicrotome.

Staining

Sections supported on copper grids were stained as follows using the method of Forsdyke (1979):

Saturated alcoholic solution of uranyl acetate, 10 min.

Wash with distilled water.

Reynold's lead citrate, 5 min.

Wash with distilled water.

Viewing sections

Stained sections were viewed with either a A.E.I.802 or JEOL 100 CX electron microscope.

Micrographs

The following micrographs have been included to illustrate both methods 1 and 2. Method 1 was used on fractions P_2A, P_2B and P_2C as prepared according to Heaton & Bachelard (1973) see Figures 4.6.1.(e)→(f). The use here of dimethoxypropane was found to cause a marked degree of vacuolation in the tissues examined. Method 2 was used on fractions P_2A; P_2B and P_2C as prepared according to Bradford et al. (1975) - see Method 5 in Table 3.1. For micrographs see Figures 4.6.1. and 4.6.1.(a)→(c). Figure 4.6.1. shows a

synaptosome illustrating all the features generally used in the identification of a synaptosome. These are (1) the presence of synaptic vesicles (De Robertis et al., 1962b), (2) an intraterminal mitochondrion, and (3) the postsynaptic thickening. When examining sections of a synaptosomal pellet the postsynaptic thickening, for example, is not always seen. As sections for electron microscopy are 500-600 Å in thickness, some 20 planes of section through the synaptosome could be visualized, a proportion of which would not include the characteristic synaptic features (Bradford, 1975). Also the unidentified membrane fragments frequently observed often turn out, by serial sectioning, to be grazed sections of synaptosomes (Whittaker, 1972).

Of the three fractions studied by electron microscopy - myelin, synaptosomes and mitochondria - the synaptosome fraction was the most difficult to prepare successfully as the preservation of the thin external membrane is particularly difficult. On exposure to cytological fixatives synaptosomes become extremely fragile (Whittaker, 1965). Thus a considerable proportion of the damage seen in fixed and stained synaptosomes is likely to be due to their fragile state during manipulation for electron microscopy rather than due to the preparation procedures. Bradford (1975) has also indicated that when serial sections are taken, a large proportion of profiles show continuity of the boundary membrane and sometimes ruptures may be seen - however these are also evident after incubation where damage to membranes by agitation may be expected.

The damage to the mitochondrial cristae seen in Figures 4.6.1.(c) and (f) is expected due to the mitochondria having been sedimented in hyperosmolar sucrose (1.2M) in the density gradient.

I have not attempted a quantitative analysis from the electron micrographs for reasons which will be described in the following section.

FIGURES 4.6.1. and 4.6.1.(a)-(f).

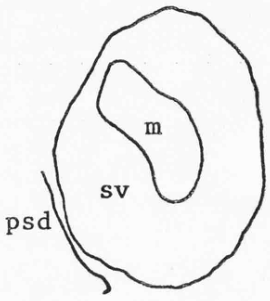
Electron micrographs have been prepared as described in Section 4.6.1. using double fixation (glutaraldehyde and osmium tetroxide) and double staining (uranyl acetate and lead citrate).

Figures (a), (b) and (c) represent subfractions of P_2 prepared by the method of Heaton & Bachelard (1973), i.e. the myelin, synaptosomal and mitochondrial fractions respectively. Tissue was prepared for electron microscopy according to Method 1.

Figures (d), (e) and (f) represent subfractions of P_2 prepared by the method of Bradford *et al.* (1975), i.e. the myelin, synaptosomal and mitochondrial fractions respectively. Fig. 4.6.1. is a synaptosome at high magnification. Tissue was prepared for electron microscopy according to Method 2.

FIGURE 4.6.1. 0.1μm ——

A typical synaptosome demonstrating the presence of an intrasynaptosomal mitochondrion (m), many synaptic vesicles (sv), and the postsynaptic density (psd).



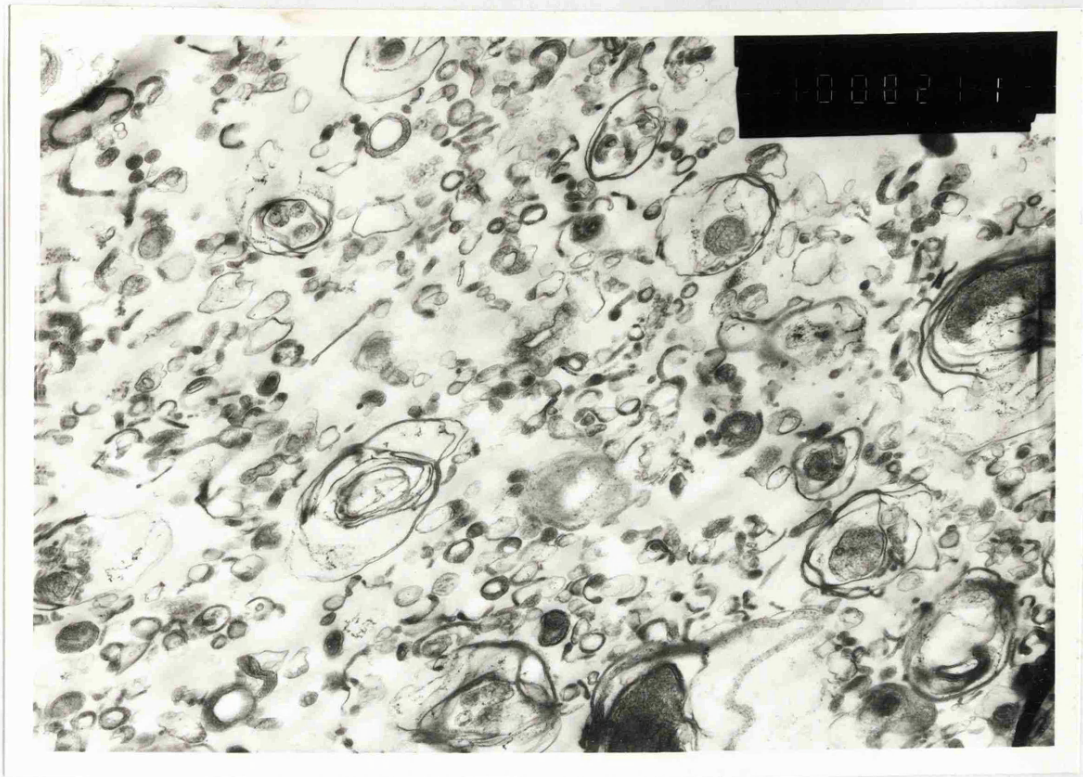


FIGURE 4.6.1.(a) MYELIN

1µm ————

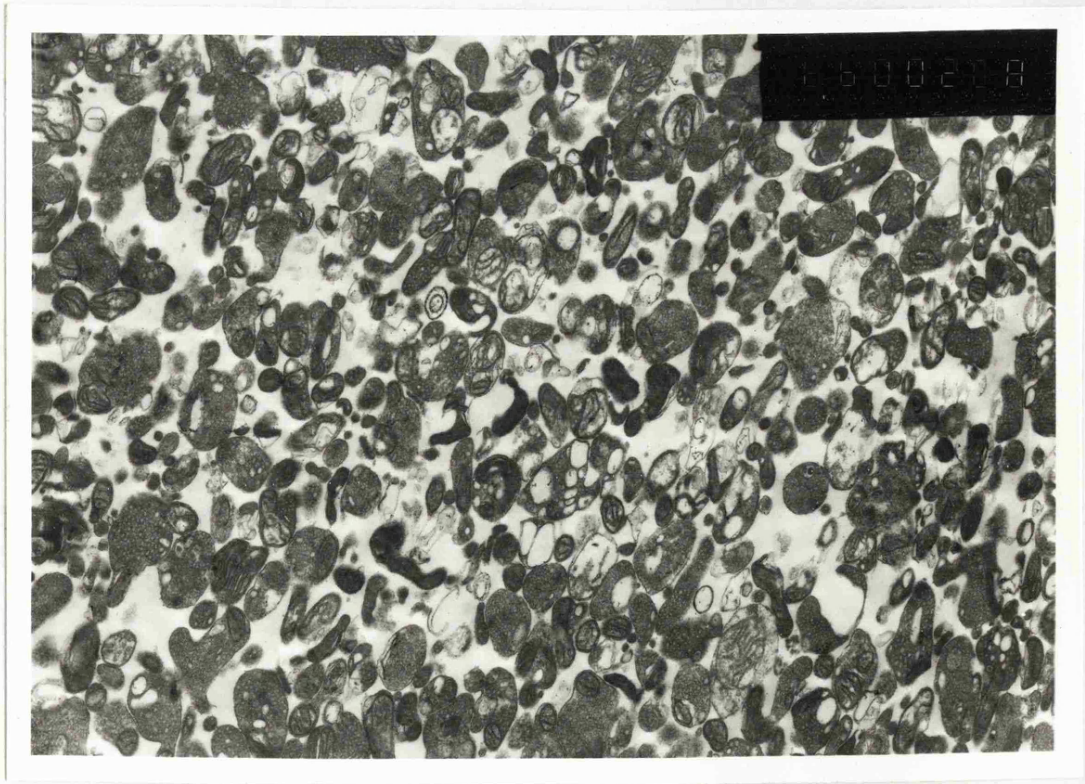


FIGURE 4.6.1.(b) SYNAPTOSOMES

1µm ————

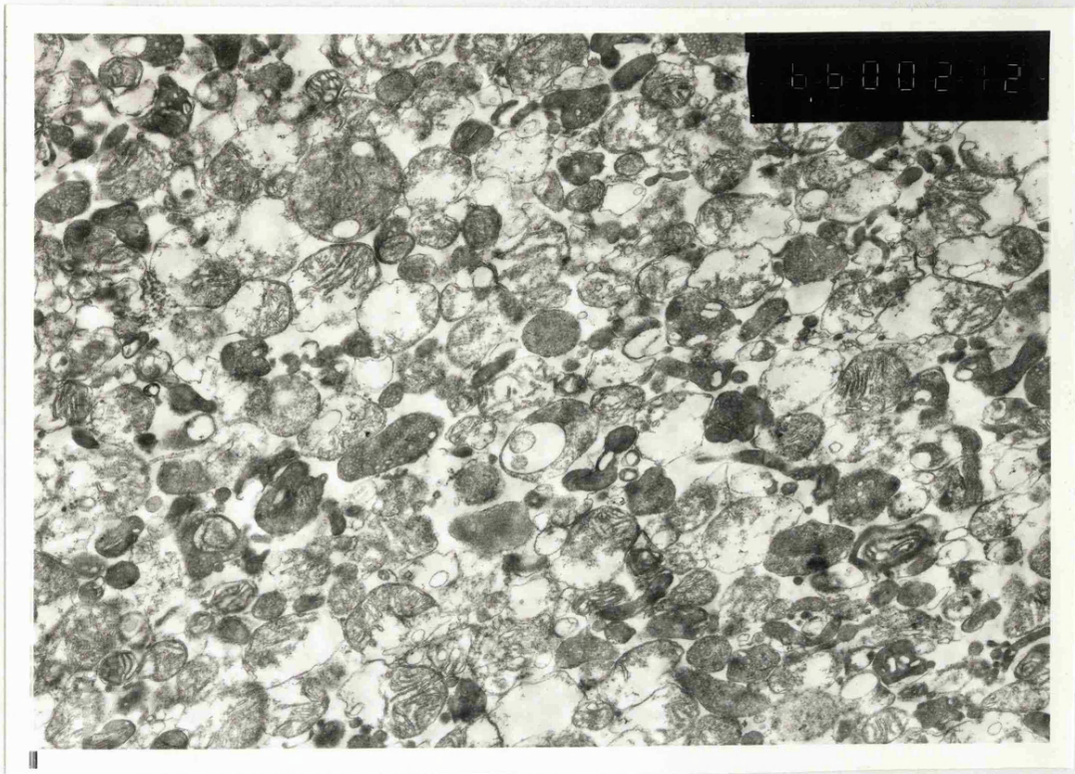


FIGURE 4.6.1.(c) MITOCHONDRIA

1µm ———

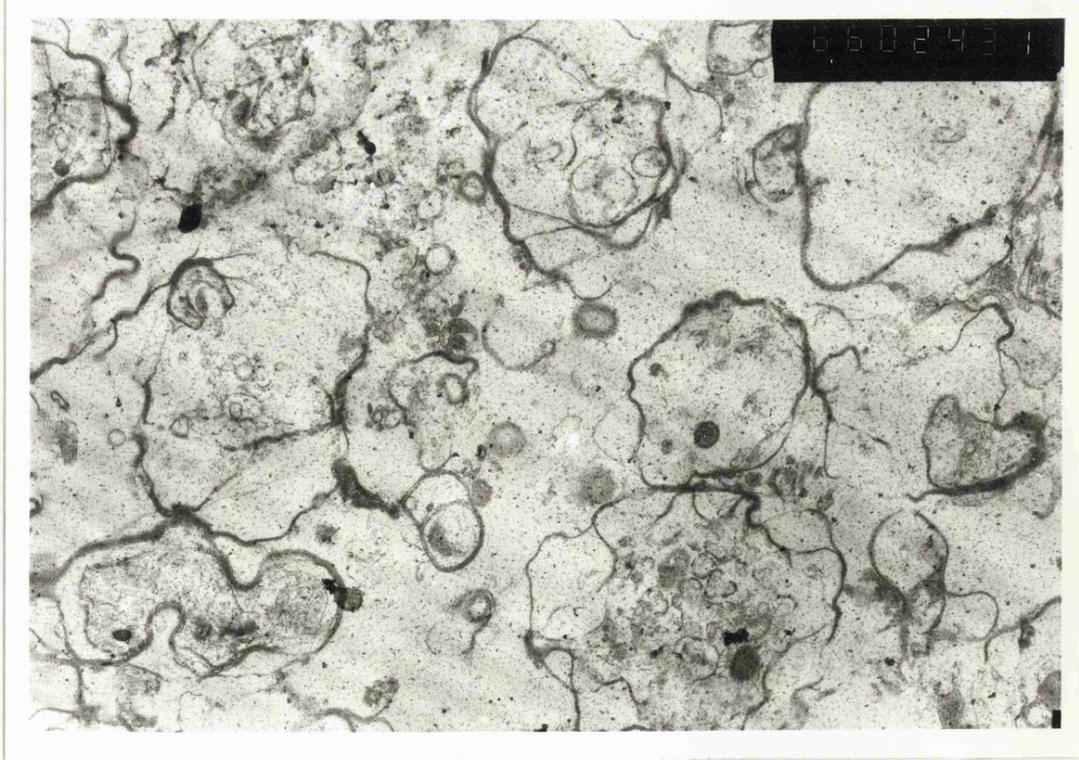


FIGURE 4.6.1.(d) MYELIN

1µm ———

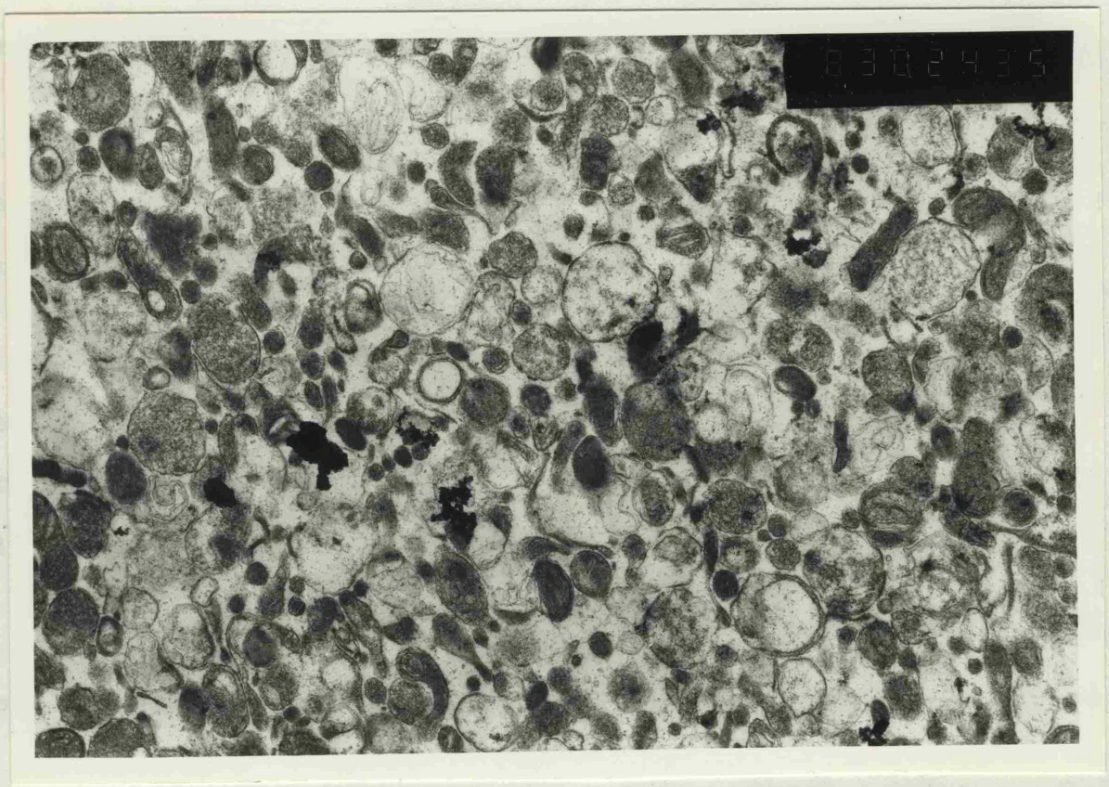


FIGURE 4.6.1.(e) SYNAPTOSOMES

1μm

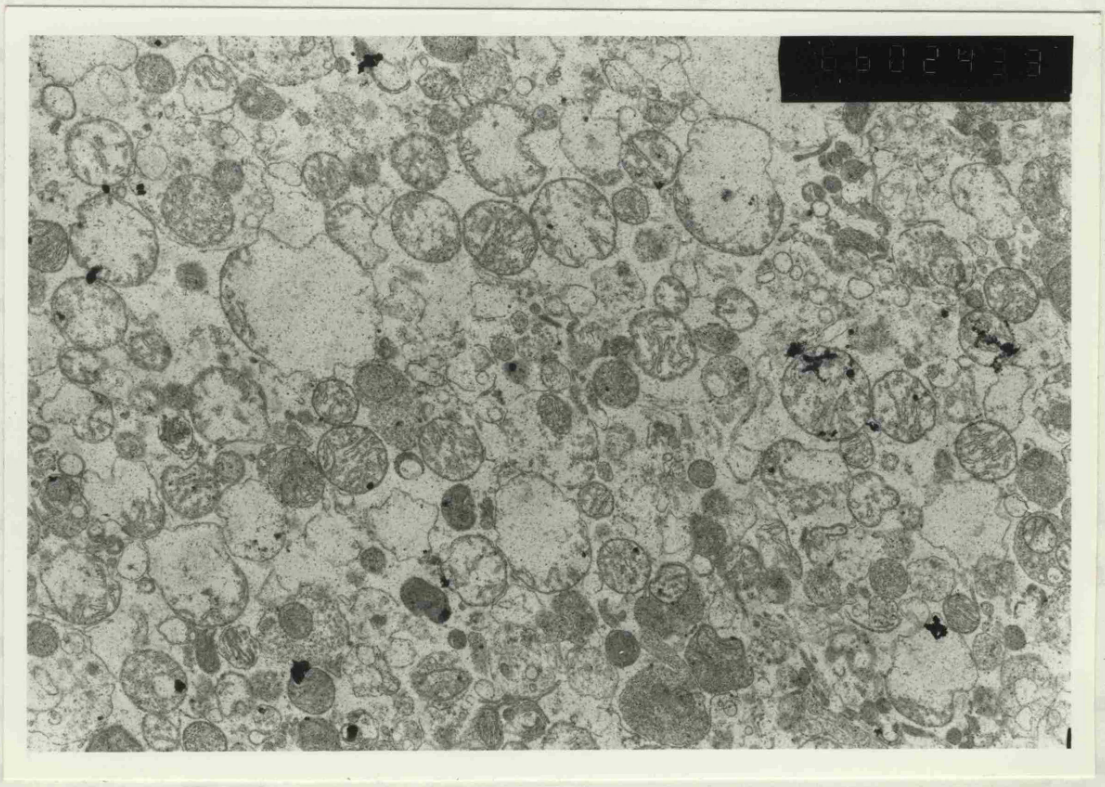


FIGURE 4.6.1.(f) MITOCHONDRIA

1μm

4.6.2. The usefulness of the electron microscope in the characterisation of subcellular fractions

Firstly two points need to be emphasized in order to put any impression of purity/contamination of subcellular fractions visualized by electron microscopy into perspective.

(a) It has been estimated that to cut serial ultrathin sections for electron microscopy through a block of tissue one cubic mm in volume, and to photograph the entire area of each section, would take approximately 4×10^8 photographic exposures (Weakley, 1972).

(b) If a $1mm^2$ section is taken from the face of such a block, in a closely packed tissue with cells averaging 15μm in diameter, profiles of over 4000 cells could be present in a single section.

Jones & Brearley (1973) have shown that subfractionation of the "synaptosome" fraction from whole brain yields lighter layers rich in axonal segments and heavier ones containing synaptosomes and axonal segments. Unless one maintains precise orientation of the sample throughout the preparation scheme before sectioning, a section taken from the top of the pellet will appear to be highly contaminated with unidentifiable membrane fragments. Similarly a section from the bottom of the pellet will suggest a preparation highly purified with synaptosomes.

Normally it is assumed that the section through the plane of sedimentation would provide a representative sample. However, centrifugation in an angle-head centrifuge, or any swinging-bucket cell with parallel walls is also affected by convective flow and no plane of section through the pellet can be expected to yield a statistically valid sample of the original fraction (Grove et al., 1973). Two methods have been described to overcome this problem, (1) the use of sector-shaped cells. Convective flow and wall interactions do not affect sedimentation in sector-shaped cells and the resulting distribution of particles is determined solely by their

sedimentation properties (Grove et al., 1973). (2) The production of disc-shaped pellets where the tissue is fixed during the centrifugation of the synaptosomal suspension. Subsequent embedding (flat) allows knowledge of orientation to be maintained (Cotman & Flansburg, 1970).

Quantification of elements in the micrograph is made by placing a grid of regularly spaced dots over a micrograph which spans the thickness of the pellet. The synaptosomal volume percentage can then be computed from the number of dots falling upon synaptosomes divided by the number of dots falling on any electron-dense object (Grove et al., 1973).

Apart from knowledge of the synaptosomal volume percentage, it is also important to have accompanying chemical and enzymic data on synaptosomes to give the fullest and most accurate picture of the composition of the fraction being studied. For example, one mitochondrion visualized per nine plasma membrane fragments contributes substantially more than 10% mitochondrial protein to the preparation since one mitochondrion contains more protein than one plasma membrane of equivalent size (Cotman, 1972).

5. THE USE OF A RANK OXYGEN ELECTRODE

5.1. Procedure for the use of a Rank oxygen electrode

The Rank oxygen electrode was obtained from Rank Brothers, Cambridge, along with an accompanying magnetic stirrer plus control unit and a voltage supply unit. See Figure 5.1. for a diagram of the apparatus.

Procedure

(a) First check that the silver anode has not been dulled by an oxidation process termed "aging" (Lessler & Brierley, 1969). If aging has occurred, the silver can be polished using commercially available silver polish, care being taken to avoid scratching the platinum cathode.

(b) Add sufficient saturated KCl to wet the silver and platinum electrodes.

(c) Cut a 1cm square of lens tissue and make a 1mm diameter hole in it with scissors. Place the tissue over the platinum electrode so that the hole is over the electrode.

(d) Cut a 1cm square piece of teflon (0.0005 inch thick, obtained from Rank Brothers) and place over the lens tissue and lock in place by screwing down the locking nut around the incubation vessel. Make sure that no air bubbles are trapped and that the membrane is not twisted.

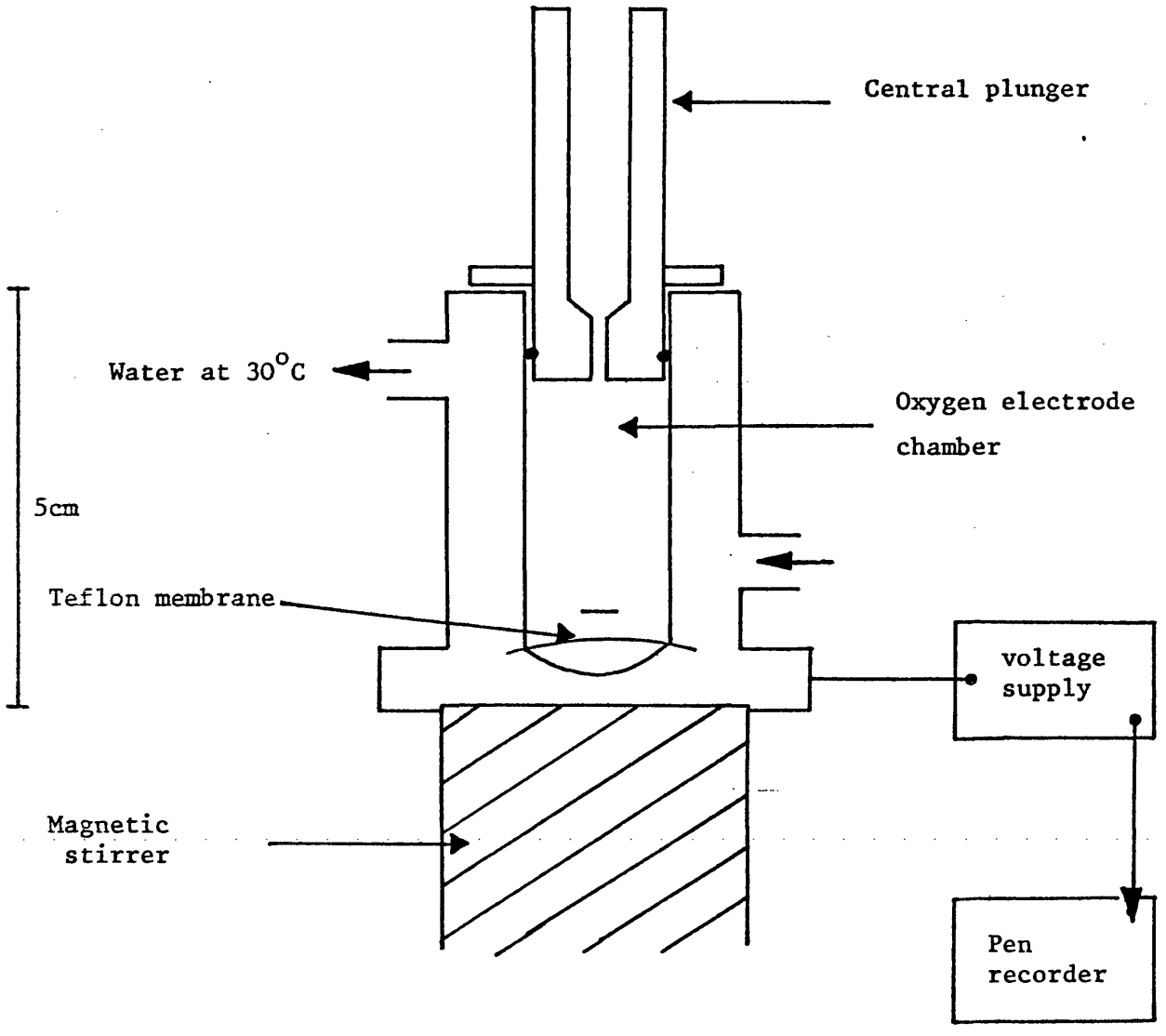
(e) Turn on the voltage supply box to polarize the platinum electrode at -0.6V with respect to the Ag-Cl.

(f) To calibrate the electrode, add 3ml of air-saturated medium at $30^{o}C$ to the oxygen electrode chamber (incubation vessel) and place the plunger in position. See Figure 5.1. It is important to ensure that no air bubbles are present. Turn on the magnetic stirrer and watch the response on the chart recorder. If the membrane has been damaged during this procedure, an unstable recording will be produced and a new membrane should be fitted.

One of the most convenient methods used to calibrate the zero

FIGURE 5.1. DIAGRAM OF RANK OXYGEN ELECTRODE

(Not to scale)



position is to add an O_2 scavenger such as a suspension of fresh yeast. The fall in oxygen content is then monitored until a steady position is reached after which the yeast suspension is washed out of the chamber using suction from a water pump with repeated additions of distilled water. With the pen now adjusted to zero, air-saturated medium is again added and the chamber is closed. The pen is adjusted to give a reading in the region of 90 units on the chart recorder paper (10mV full scale).

(g) Any additions to the oxygen electrode chamber can be made from a Hamilton syringe through a small hole in the centre of the plunger. Care must always be taken not to introduce air bubbles.

Solubility of oxygen in the media used

The solubility of oxygen in Ringers solution at 30°C = 0.026 ml O_2/ml fluid (Umbreit *et al.*, 1972). 0.026 is the Bunsen solubility coefficient (α) for the conditions as defined.

The following calculations determine the concentration of oxygen in solution.

If 1 mole O_2 = 22.4ℓ at STP,

then at 30°C, 1 mole O_2 = 24.86ℓ.

Therefore there are $\frac{26}{24.86}$ μmoles O_2/ml fluid

= 1.05 mM O_2

But as P_{O_2} is approximately 21% of atmosphere pressure, then the concentration of O_2 in air-saturated fluid is (1.05mM x $\frac{21}{100}$) = 0.220mM.

21% O_2 ≡ 158.8 torr

158.8 mmHg ≡ 0.220mM O_2 in solution at 30°C.

In order that the degree of comparability between experiments is maximal, various factors influencing tissue and electrode responses must be kept constant. These are for example, (1) temperature - the contents of the

oxygen electrode chamber are kept constant at 30°C by water circulating through a water jacket. The medium being used is also kept at 30°C. (2) Stirring rate - this must be kept constant for two reasons, (a) stirring may produce a degree of cellular damage, and (b) any transfer of oxygen from the atmosphereto the medium, or vice versa for low oxygen tension experiments, will depend on the rate of stirring. The stirring rate must be maintained sufficient to prevent depletion of oxygen content in the layer of solution near the electrode. (3) Where possible, the same ratio of tissue volume to medium volume should be maintained.

It should be remembered that the electrode itself consumes oxygen at a constant rate from the solution. The significance of this electrode error will depend on the rate of oxygen consumed by the sample. The electrode error will become an important factor when using systems that use oxygen slowly, therefore it is advisable to increase the quantity of biological material so that the reaction requires a shorter period of time for measurement.

The rate of loss of oxygen from the oxygen electrode chamber containing air-saturated medium is 0.18 μmoles O_2/h (S.E.M., 0.02; n = 9). There is no significant difference in this rate in the presence of 10mM glucose or bovine serum albumin at 3.33mg/ml.

5.2. Conversion of the Rank oxygen electrode for use as an oxystat

The simplest design for a piece of apparatus capable of maintaining a constant oxygen environment for a respiring tissue requires a system to measure the oxygen tension, calculate its deviation from the required oxygen tension and add sufficient oxygen to counteract this deviation. An automated design for an oxystat has been described by Jones & Mason (1978) and this provides the basis for the adaptation of the Rank oxygen electrode used here.

The central plunger (see Figure 5.1.) is not used for these experiments. The gas is blown through a wide-bore glass tube over the stirred surface of the solution within the oxygen electrode chamber. The system is closed by wrapping Parafilm around the glass tube and over the top of the chamber. Varying oxygen concentrations in the gas phase are produced from a gas mixer consisting of two flowmeter tubes connected to a glass mixing bulb. The gases used are air and nitrogen. The oxygen tension in the solution is monitored on the chart recorder and is set close to the required value by manually altering the proportions of air to N_2. Synaptosomes are added to the solution through the Parafilm by using a Hamilton syringe and care is taken to make sure that no air bubbles are introduced. A balance is then achieved between the rate at which the tissue depletes the oxygen content of the medium and the rate at which oxygen transfers from the gas phase to the liquid phase. Rapid manual alteration of the gas phase is hampered by the dead space between the gas cylinders and the gas outlet over the suspension. However practice helps to reduce any deviations from the required set point.

6. THE INFLUENCE OF VARIOUS MEDIA AND SUBSTRATES ON SYNAPTOSOMAL OXYGEN UPTAKE

The principle report in the literature involving the use of an oxygen electrode for measuring oxygen uptake by synaptosomes was by Verity (1972). The following describes experiments to verify his conclusions and thus provide a basis for further research.

6.1. METHODS

6.1.1. Preparation of synaptosomes

Rat cerebral cortex synaptosomes were prepared by Method 3 as described in Section 3.

6.1.2. Media

Two different media were used in the following experiments:

(a) Verity's medium (Verity, 1972).

Composition (mM): KCl, 100; KH_2PO_4, 5; K-EGTA, 1; and tris-HCl, 20 at pH 7.4.

(b) Krebs phosphate medium (Bradford et al., 1975).

Composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 0.75; $MgSO_4$, 1.3; Na_2HPO_4, 20 at pH 7.8.

6.1.3. Experimental design

To the oxygen electrode chamber was added 2.9ml of either medium at 30°C. The medium was fully saturated with air that had previously been passed through a gas-wetter. When the chart recorder trace had stabilised at a level representing an oxygen tension of 158.8mmHg (see Section 5.1.), a 100µl sample of synaptosomes, resuspended in that same medium, was added using a Hamilton syringe. On some occasions 200µl samples were used but the final volume always remained 3ml.

100µl of resuspended synaptosomal pellet is equivalent to 1.68mg protein (S.E.M., 0.50; n = 32). Samples were kept on ice until required.

Further additions to the oxygen electrode chamber were made in volumes of 10-50µl. Oligomycin and DNP were dissolved in ethanol, all other substances were dissolved in the medium used.

6.2. RESULTS

Since the rate of synaptosomal oxygen uptake was found to decline slowly during the course of an experiment, absolute values in terms of the rate of oxygen uptake do not always give a clear indication of the effect of the added substrate. For this reason results are also expressed as the ratio of the rate after the addition to the rate before the addition of the substrate under test.

6.2.1. The effect of exogenous ADP on glutamate-supported oxygen uptake by synaptosomes

The following Table 6.2.1. shows that the addition of 0.5 µmoles of ADP to a suspension of synaptosomes in the presence of 5mM glutamate stimulates oxygen uptake (Verity's medium).

TABLE 6.2.1. The effect of exogenous ADP on glutamate-supported oxygen uptake by synaptosomes at 30°C in Verity's medium

Additions	n	Rate of O_2 uptake		Ratio
		No ADP	ADP	
5mM glutamate + 5mg BSA	7	39.35(4.82)	59.63(6.31)	1.68(0.17)
5mM glutamate + 10mg BSA	16	32.73(1.75)	54.10(3.20)	1.66(0.05)

Values for respiratory activity are expressed as µmoles O_2/hr/100mg protein; Mean (S.E.M.)

Although ADP significantly stimulates oxygen uptake, the increased rate takes a few minutes to become established and slows down very gradually. This is in contrast to the rapid on-off response observed by Verity (1972). There is no significant difference here between the presence of 5 or 10mg BSA (bovine serum albumin).

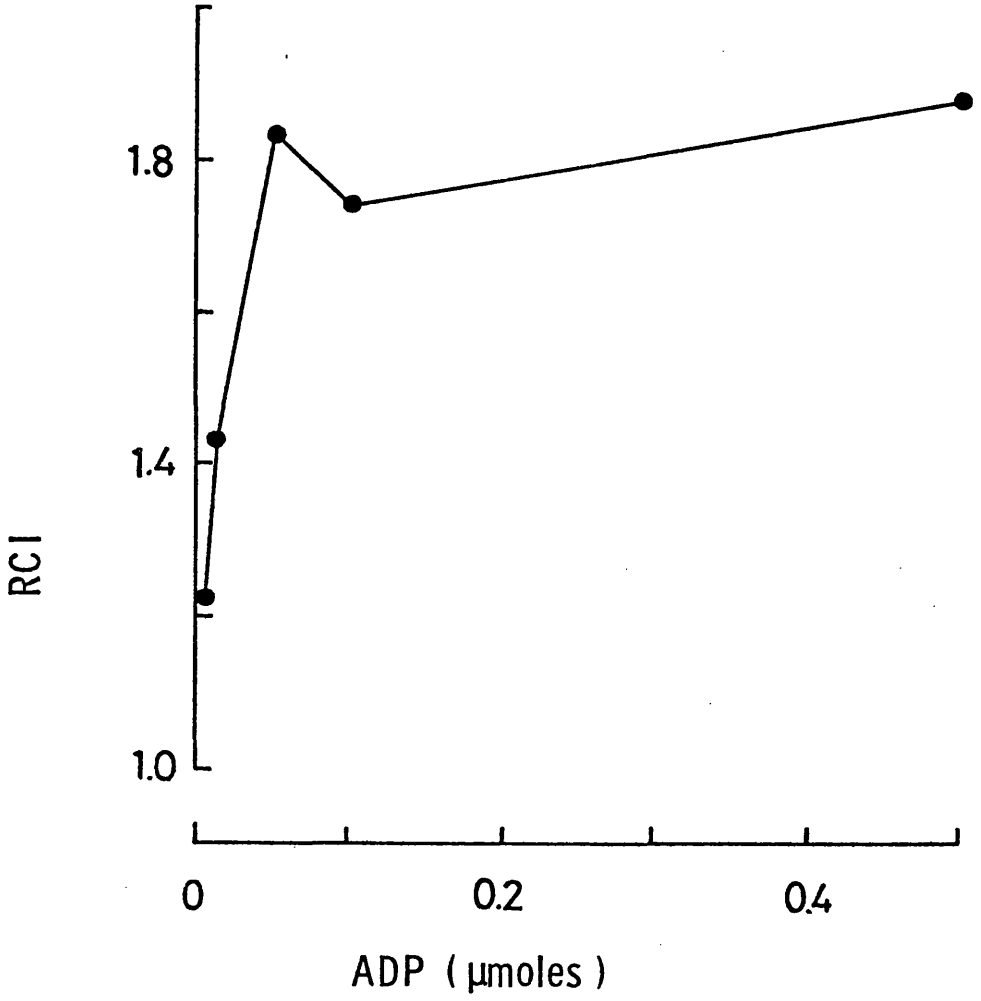
6.2.2. The effect of varying concentrations of ADP on synaptosomal oxygen uptake

Verity (1972) has considered that the addition of ADP can be viewed in terms of State 3 respiration as defined by Chance & Williams (1955). State 3 respiration occurs in the presence of substrate and a phosphate acceptor (ADP) and allows the rapid utilization of oxygen to occur. When all available ADP has been phosphorylated, respiration will become slow. This is termed State 4 and is the "state of respiratory control".

The respiratory control index (RCI) is defined as the ratio of the respiratory rate in the presence of ADP to that in its absence and is a measure of mitochondrial metabolic integrity. Loss of respiratory control is shown by an increased rate of mitochondrial oxygen utilization in the absence of ADP (State 4).

Using an alternative substrate (5mM succinate), the amount of ADP added was varied to determine if the 0.5 μmoles ADP used was the limiting factor in distinguishing State 3 and State 4 respiration. The results are shown in Figure 6.2.2. and indicate that 0.5 μmoles ADP is optimal for stimulation of oxygen uptake by synaptosomes. Once again these results in the presence of succinate do not correspond to the high RCI values of Verity (1972).

FIGURE 6.2.2. THE EFFECT OF VARYING CONCENTRATIONS OF ADP ON THE 'RESPIRATORY CONTROL INDEX' OF SYNAPTOSOMES



Measured at 30°C in Verity's medium containing BSA (3.33mg/ml) and succinate (5mM). Final volume 3ml.

6.2.3. Comparison of the effects of exogenous ADP on synaptosomal oxygen uptake supported by glutamate, succinate and glucose

The results are summarized in Table 6.2.3. and indicate that only succinate is capable of stimulating synaptosomal oxygen uptake. However both succinate and glutamate are able to support ADP stimulation of oxygen uptake.

TABLE 6.2.3. Comparison of the effects of exogenous ADP on synaptosomal oxygen uptake supported by glutamate, succinate and glucose

Substrate	n.	Rate of O_2 uptake	Ratio before and after addition	Degree of significance
(a) Addition of substrates				
4mM glutamate	15	19.69(2.01)	1.09(0.08)	NS
5mM glutamate	4	32.63(3.89)	1.10(0.12)	NS
4mM succinate	3	46.27(5.54)	2.24(0.27)	P<0.001
5mM succinate	4	54.51(1.97)	2.45(0.48)	P<0.01
10mM glucose	3	30.85(2.83)	0.94(0.03)	NS
(b) Addition of 0.5 µmoles ADP in the presence of				
4mM glutamate	15	34.64(2.87)	1.86(0.16)	P<0.001
5mM glutamate	3	51.29(3.06)	2.30(0.33)	P<0.05
4mM succinate	3	78.84(10.39)	1.71(0.13)	P<0.01
5mM succinate	5	123.37(19.40)	1.62(0.24)	P<0.05
None	2	40.72(4.38)	1.25(0.10)	NS

Measured at 30°C in Verity's medium containing BSA (3.33mg/ml).

Values for respiratory activity are expressed as µmoles O_2/hr/100mg protein; Mean (S.E.M.).

6.2.4. Comparison of results with those of Verity (1972).

A direct comparison can be made with the results obtained by Verity (1972) when considering glutamate-supported oxygen uptake, see Table 6.2.4.

TABLE 6.2.4. Comparison of results with those of Verity (1972)

Experiments	Substrate	n.	Respiration rates State 4	State 3	*RCI
Verity	4mM glutamate	7	0.88 [0.6–1.6]	4.9 [3.3–6.5]	4.9
Wise	4mM glutamate	15	0.66(0.07)	1.14(0.09)	1.86(0.16)

* Calculated from individual experiments.

Measured at 30°C in Verity's medium containing BSA (3.33mg/ml).

Values for respiratory activity are expressed as µg atoms of O/min/100mg protein, Mean (S.E.M.). Square brackets indicate the range of results.

These results clearly show that the addition of ADP to my synaptosomal preparation fails to elicit the same magnitude of response as stated by Verity (1972).

6.2.5. The effect of 2,4-dinitrophenol and oligomycin on synaptosomal oxygen uptake

If synaptosomal oxygen uptake is a reflection of mitochondrial respiration, it should be possible to block the ADP stimulated oxygen uptake by the addition of oligomycin which at low concentrations preferentially inhibits the mitochondrial ATPase involved in oxidative phosphorylation.

It should also be possible to overcome this blockade by the addition of an uncoupling agent, 2,4-dinitrophenol (DNP) which dissociates ATP synthesis from respiration. In the presence of DNP, mitochondrial oxygen consumption occurs at a high rate but no ATP is produced.

To test this assumption, additions were made in the following order to the oxygen electrode chamber: 100µl synaptosomes (1.68mg protein), 4mM glutamate, 0.5 µmoles ADP, 18µM oligomycin, 0.5 µmoles ADP, and 100µM DNP. Figure 6.2.5. gives as an example, the results of an experiment as described above.

In all cases, DNP overcame the block on ADP stimulation due to oligomycin. Oligomycin decreases the rate of oxygen uptake to the control rate in the absence of ADP. Ratio of rates:- oligomycin/synaptosomes plus glutamate = 1.11 (S.E.M., 0.11; n = 7; No significant difference).

Table 6.2.5. shows a combination of these experiments. DNP produced a stimulation of oxygen uptake of almost twice the magnitude observed on the addition of ADP to the synaptosome suspension in the presence of glutamate.

TABLE 6.2.5. The effect of 2,4-dinitrophenol on synaptosomal oxygen uptake

Addition (In order added)	n.	Rate of O_2 uptake	Ratio	Degree of significance
Synaptosomes	33	23.41(1.78)		
4mM glutamate	15	19.69(2.01)	1.09(0.08)	NS
0.5 µmoles ADP	15	34.64(2.87)	1.86(0.16)	P<0.001
100µM DNP	5	62.74(6.09)	1.99(0.20)	P<0.01

Measured at 30°C in Verity's medium containing BSA (3.33mg/ml). The rate given for DNP is after the addition of oligomycin and ADP as described in the text. Values for respiratory activity are expressed as µ moles O_2/hr/100mg protein; Mean (S.E.M.).

FIGURE 6.2.5. THE EFFECT OF 2,4-DINITROPHENOL AND OLIGOMYCIN ON SYNAPTOSOMAL OXYGEN UPTAKE

Fig. 6.2.5. is a direct copy of a single experiment as recorded by the pen recorder.

Measured at 30°C in Verity's medium containing BSA (3.33mg/ml). The quantities used in a final volume of approx. 3ml were synaptosomes (1.68mg protein), glutamate (4mM), ADP (0.5µmoles), oligomycin (18µM) and DNP (100µM).

The ratio values stated represent the ratio of the rate of synaptosomal oxygen uptake after the specified addition to the rate before the addition.

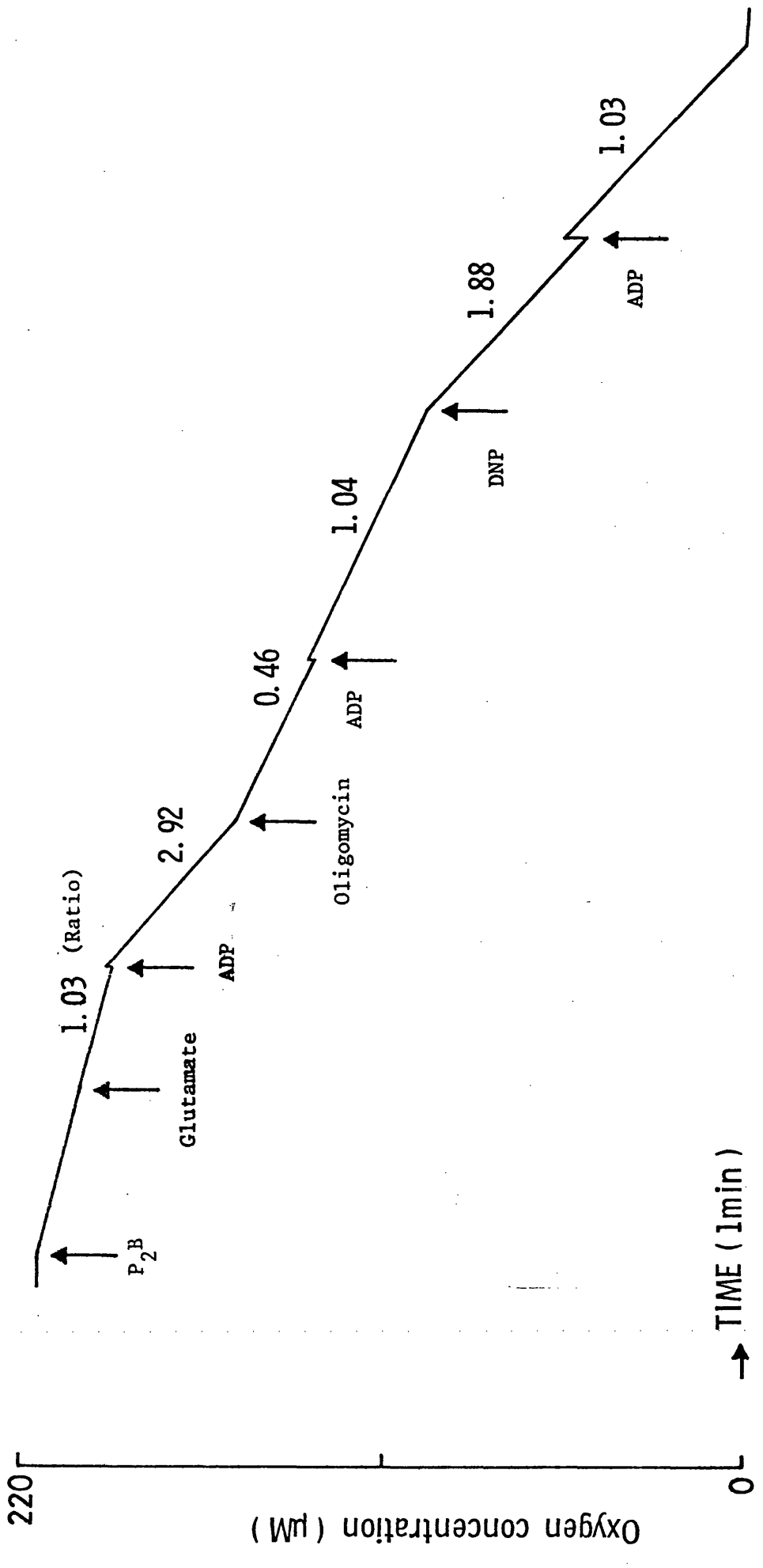

220
0
Oxygen concentration (µM)
TIME (1min)
P_2^B
1.03 (Ratio)
Glutamate
2.92
ADP
0.46
Oligomycin
1.04
ADP
1.88
DNP
1.03
ADP

FIGURE 6.2.5.

These results show that although synaptosomes respond poorly to the addition of ADP (See Section 6.2.3.), they do exhibit responses reflecting the presence of mitochondria as shown by the DNP-stimulated oxygen uptake in the presence of oligomycin.

6.2.6. The effect of increasing osmotic shock on synaptosomal oxygen uptake

Since the synaptosomes have been shown to have some properties reflecting the presence of intrasynaptosomal mitochondria, it may be that the synaptosomal limiting membrane is not readily permeable to ADP.

During the preparative procedures, synaptosomes taken from the density gradient are diluted x2 with distilled water to restore isotonic conditions. By increasing the dilution factor the synaptosomes, which respond by volume change to alterations in the tonicity of the suspension fluid (Keen & White, 1970), will undergo hypotonic shock. Increasing the degree of hypotonic shock will render the outer limiting membrane leaky until with sufficient dilution, the synaptosomes will burst.

These experiments involve the same procedures as described in Section 6.2.5. and the results are tabulated in Table 6.2.6. See also Figure 6.2.6. for a visual representation of the data.

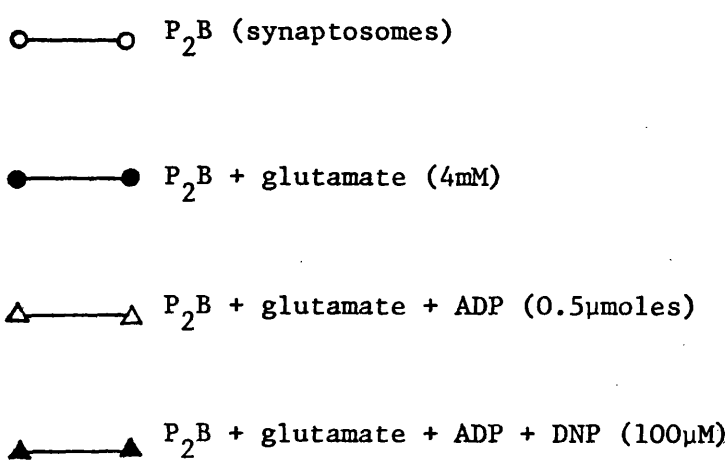
The results suggest that ADP translocation across the synaptosomal limiting membrane is not the limiting factor for production of a respiratory control index equivalent to that stated by Verity (1972).

TABLE 6.2.6. The effect of increasing osmotic shock on synaptosomal oxygen uptake

Dilution	Addition (in order added)	n.	Rate of O_2 uptake	Ratio	Degree of significance
Control x2	Synaptosomes	33	23.41(1.78)		
x4		7	12.77(2.01)		
x6		5	11.08(1.16)		
x8		6	11.28(1.56)		
Freeze/thaw		2	7.12(1.56)		
x2	4mM glutamate	15	19.69(2.01)	1.09(0.08)	NS
x4		7	13.39(1.83)	1.08(0.07)	NS
x6		5	12.39(1.21)	1.16(0.09)	NS
x8		6	12.16(2.16)	1.06(0.04)	NS
Freeze/thaw		2	8.21(0.49)	1.14(0.17)	NS
x2	0.5µmoles ADP	15	34.64(2.87)	1.86(0.16)	P<0.001
x4		7	25.35(4.84)	1.66(0.16)	P<0.01
x6		5	24.49(6.12)	1.89(0.33)	P<0.05
x8		6	24.30(6.48)	1.92(0.40)	P<0.05
Freeze/thaw		2	10.31(0.22)	1.17(0.15)	NS
x2	100µM DNP	5	62.74(6.09)	1.99(0.20)	P<0.01
x4		5	58.35(6.38)	2.40(0.37)	P<0.01
x6		5	27.29(3.27)	1.47(0.11)	P<0.01
x8		4	18.97(6.41)	1.10(0.05)	NS
Freeze/thaw		2	11.59(1.50)	1.16(0.14)	NS

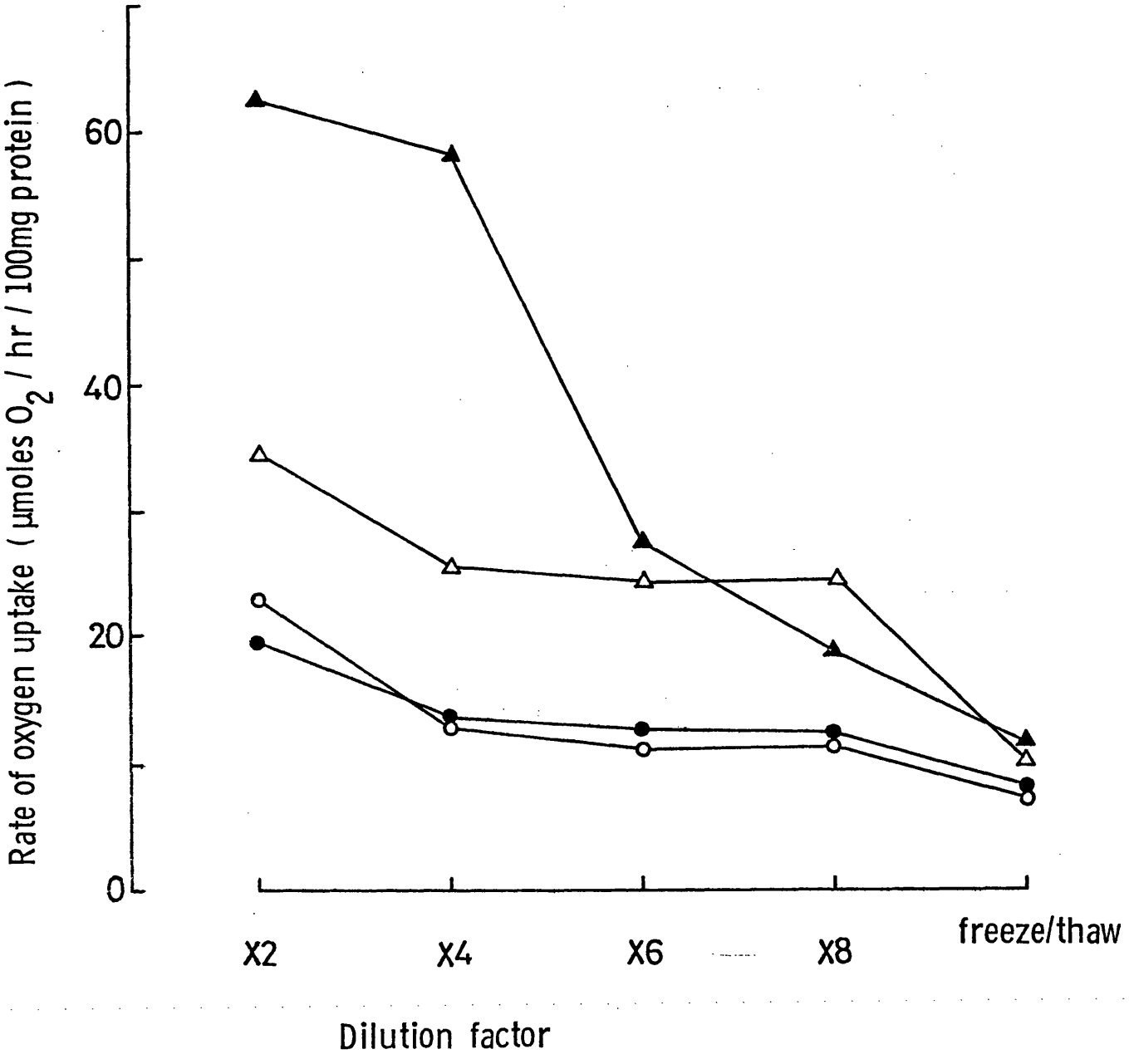
Measured at 30°C in Verity's medium containing BSA (3.33mg/ml). The rate given for DNP is after the addition of oligomycin and ADP as described in Section 6.2.5. Values for respiratory activity expressed as µmoles O_2/hr/100mg protein; Mean (S.E.M.).

FIGURE 6.2.6. THE EFFECT OF INCREASING OSMOTIC SHOCK ON SYNAPTOSOMAL OXYGEN UPTAKE



Measured at 30°C in Verity's medium containing BSA (3.33mg/ml). The rate given for DNP is after the addition of oligomycin and ADP as described in Section 6.2.5. Final volume 3ml. (For further data see Table 6.2.6.).

FIGURE 6.2.6.


Rate of oxygen uptake (µmoles O_2 / hr / 100mg protein)
60
40
20
0
X2
X4
X6
X8
freeze/thaw
Dilution factor

6.2.7. The effect of Na^+ ions on synaptosomal oxygen uptake

Verity (1972) reported a stimulatory effect of Na^+ on synaptosomal oxygen uptake which he concluded to be due to increased availability of ADP by stimulation of the synaptosomal membrane Na^+, K^+-ATPase.

Preliminary experiments using glutamate, succinate, glucose, or no substrate, suggested that the addition of 40mM Na^+ caused an increase in oxygen uptake.

Verity (1972) noted Na^+ stimulation was more consistently seen after a priming ADP phosphorylation cycle and that ouabain could block the Na^+ stimulation of respiration. Despite the low number of observations here, the results in Table 6.2.7. indicate that the Na^+ stimulation was not as marked as seen by Verity (1972) but that this stimulation could also be reversed by the addition of ouabain.

TABLE 6.2.7. The effect of Na^+ ions on synaptosomal oxygen uptake

Additions (In order added)	n.	Rate of O_2 uptake	Ratio
4mM glutamate			
0.5μmoles ADP	2	28.76(2.18)	
40mM Na^+	2	39.02(1.07)	1.36(0.07)
180μM ouabain	2	29.12(4.03)	0.74(0.08)

Measured at 30°C in Verity's medium containing BSA (3.33mg/ml). Values for respiratory activity are expressed as μmoles O_2/hr/100mg protein; Mean (S.E.M.)

6.2.8. Preparation of synaptosomes by the method of Verity (1972)

Rat cerebral cortex synaptosomes were prepared according to the method described by Verity (1972) but with the usual homogenization procedure as described in Method 3, see Section 3.1. This preparation failed to produce ADP stimulation comparable to that observed by Verity (1972). The more gentle homogenization he used may reduce the efficient conversion of intact nerve terminals to synaptosomes and thus produce an increased proportion of larger structures such as mitochondria. His short centrifugation times would increase the proportion of mitochondria in fraction P_2 and prevent efficient separation on the density gradient, see Figure 6.2.8. The high activities of malate dehydrogenase and cytochrome c oxidase in his synaptosomal fraction compared to the mitochondrial fraction suggest that there could be a high degree of mitochondrial contamination which would be responsible for his very clear distinction between State 3 and State 4 respiration.

6.2.9. Oxygen uptake by the crude mitochondrial fraction (P_2)

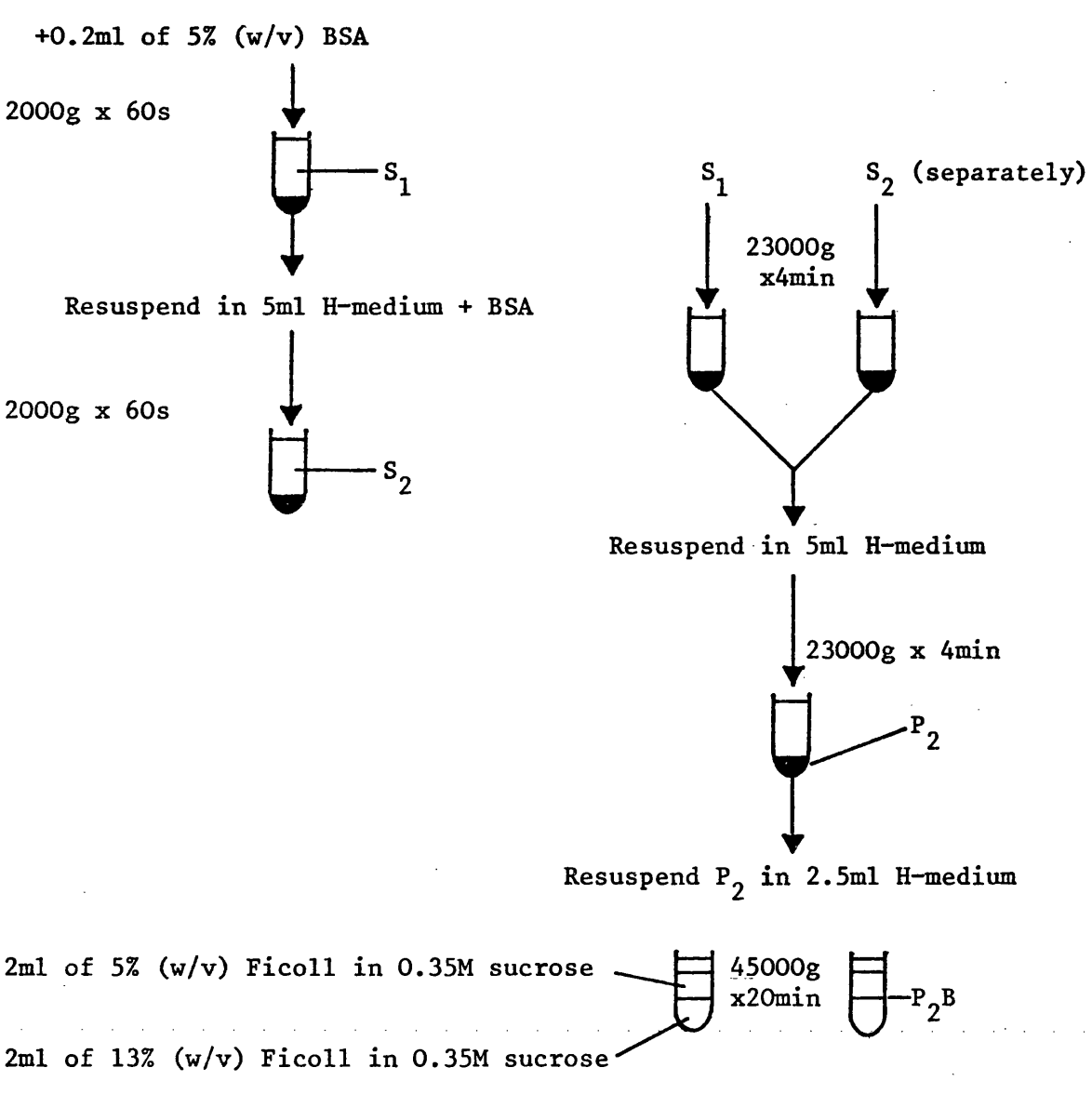
To confirm whether or not the transition between State 3 and State 4 respiration observed by Verity (1972) was due to mitochondrial contamination, oxygen uptake was measured in the crude mitochondrial fraction (myelin, and synaptosomes plus mitochondria). Preliminary experiments indicated that 0.5μmoles ADP was optimal for stimulation of oxygen uptake, see Figure 6.2.9.

Additions of 5mM glutamate, 0.5μmoles ADP and 40mM Na^+ were made and the results are summarized in Table 6.2.9.(i).

FIGURE 6.2.8. PREPARATION OF SYNAPTOSOMES BY THE METHOD OF VERITY (1972)

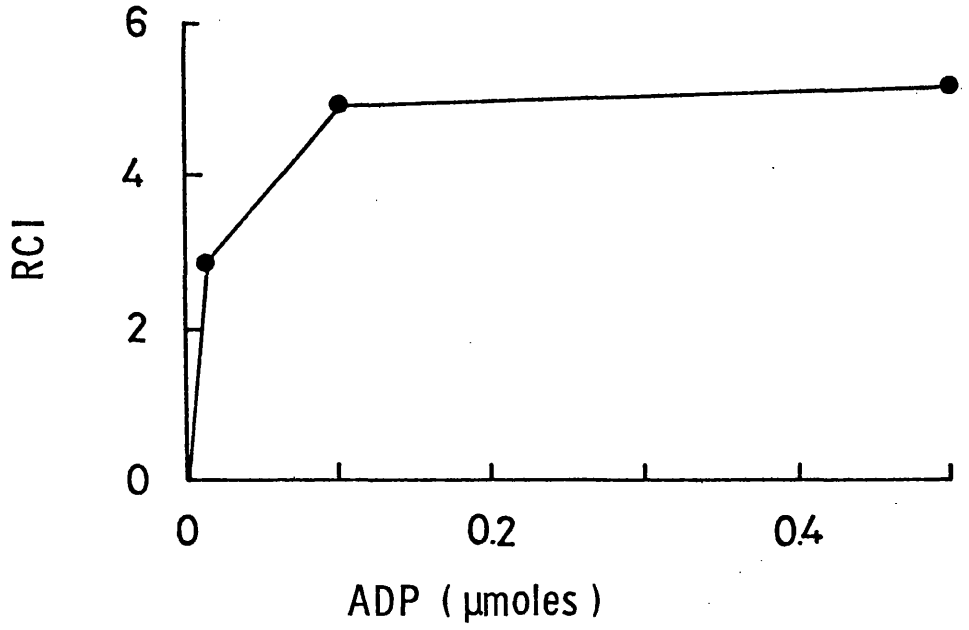
Homogenization medium: 0.35M sucrose, 10mM tris-HCl (pH7.4)
0.5mM K-EGTA

All procedures at 0-4°C



Synaptosomes (P_2B) are collected and diluted with H-medium to 1 or 2ml.

FIGURE 6.2.9. THE EFFECT OF VARYING CONCENTRATIONS OF ADP ON THE 'RESPIRATORY CONTROL INDEX' OF P_2 (CRUDE MITOCHONDRIAL FRACTION)



Measured at 30°C in Verity's medium containing BSA (3.33mg/ml) and glutamate (5mM).
Final volume 3ml.

TABLE 6.2.9.(i). Oxygen uptake by the crude mitochondrial fraction

	Addition	n.	Rate of O_2 uptake	Ratio
(a)	No substrate			
	None	2	13.65(0.17)	
	0.5µmoles ADP	2	28.39(0.09)	2.08(2.02)
(b)	Substrate supported O_2 uptake			
	5mM glutamate	10	15.41(1.62)	
	0.5µmoles ADP	7	77.76(9.54)	5.17(0.33)
(c)	Addition of 40mM Na^+ after ADP cycle			
	5mM glutamate	2	15.63(0.81)	
	0.01µmoles ADP	2	44.86(9.11)	2.85(0.44)
	End ADP cycle	2	14.02(1.72)	
	40mM Na^+	2	23.96(4.51)	1.70(0.12)

Measured at 30°C in Verity's medium containing BSA (3.33mg/ml). Additions were made in the descending order stated. Values for respiratory activity are expressed as µmoles O_2/hr/100mg protein; Mean (S.E.M.).

The results in Table 6.2.9.(i) indicate that ADP significantly stimulates oxygen uptake by fraction P_2 (crude mitochondrial pellet), both in the presence and absence of exogenous substrate, glutamate. The results also suggest that Na^+ can also stimulate oxygen uptake by this fraction.

A comparison of these results with those of Verity (1972) is detailed in the following Table 6.2.9.(ii).

TABLE 6.2.9.(ii). Comparison of oxygen uptake by the crude mitochondrial fraction with the data of Verity (1972)

Experiments	Substrate	n.	Respiration rates State 4	State 3	*RCI
Verity	4mM glutamate	7	0.88 [0.6-1.6]	4.9 [3.3-6.5]	4.9
Wise	5mM glutamate	7	0.51(0.08)	2.59(0.32)	5.17(0.33)

* Calculated from individual experiments.
Measured at 30°C in Verity's medium containing BSA (3.33mg/ml). Values for respiratory activity are expressed as µg atoms of O/min/100mg protein, Mean(S.E.M.). Square brackets indicate the range of results.

The low values for oxygen uptake by the crude mitochondrial pellet is probably due to the protein contribution by the myelin present which has no respiratory activity and therefore gives an underestimate of the true value. However calculation of the RCI overcomes this problem and gives a value that is far more comparable with the data of Verity (1972). This strongly suggests a marked degree of mitochondrial contamination in his synaptosome preparation.

6.2.10. The effect of various substrates on synaptosomal oxygen uptake in Krebs phosphate medium. Preliminary experiments.

Preliminary experiments suggested that 5mM succinate was unable to stimulate oxygen uptake by synaptosomes in Krebs phosphate medium, a result also seen with 4mM glutamate and 10mM glucose.

18µM oligomycin again inhibited oxygen uptake (see Section 6.2.5.) producing a block which could be overcome by DNP but not by ADP.

55mM K^+ was added to two samples and found not to affect the rate of oxygen uptake to any marked degree. These results are given in Table 6.2.10.

TABLE 6.2.10. The effect of various substrates on synaptosomal oxygen uptake in Krebs phosphate medium. Preliminary experiments.

Addition	n.	Rate of O_2 uptake	Ratio
(a) Addition of substrate			
None	2	59.15(2.93)	
4mM glutamate		51.01(1.70)	0.87(0.08)
5mM succinate	1		1.03
10mM glucose	1		1.04
(b) Addition of 55mM K^+			
10mM glucose	1	74.73	
55mM K^+		66.06	0.88
5mM succinate	1	82.20	
0.5µmoles ADP		88.07	1.07
55mM K^+		83.49	0.95
(c) Addition of ADP and DNP			
None	2	59.15(2.93)	
4mM glutamate		51.01(1.70)	0.87(0.08)
0.5µmoles ADP		48.42(12.48)	0.95(0.22)
100µM DNP		158.21(45.78)	1.33(0.94)

Measured at 30°C in Krebs phosphate medium containing BSA (3.33mg/ml). In (c) the rate with DNP is after the addition of oligomycin and ADP as described in Section 6.2.5. Values for respiratory activity are expressed as µmoles O_2/hr/100mg protein; Mean (S.E.M.). Square braces mean sequential addition.

6.2.11. The effect of bovine serum albumin on the uptake of oxygen by synaptosomes in Krebs phosphate medium

Verity (1972) reported that the inclusion of BSA in synaptosomal suspensions was obligatory in providing optimal respiratory conditions. To test this hypothesis, two series of experiments were performed in the absence and presence of 10mg BSA and 200µl samples of synaptosomes (∿ 3.36mg protein) in a final volume of 3ml Krebs phosphate medium. Additions of 10mM glucose, 0.5µmoles ADP and 100µM DNP were made in the order stated and the results are given in Table 6.2.11.

TABLE 6.2.11. The effect of bovine serum albumin on the uptake of oxygen by synaptosomes in Krebs phosphate medium

Addition (In order added)	n.	Rate of O_2 uptake	Ratio
(a) In the absence of BSA			
Synaptosomes	7	63.30(8.98)	
10mM glucose	8	56.81(6.66)	0.91(0.02)
0.5μmoles ADP	6	54.69(7.96)	1.01(0.02)
100μM DNP	7	144.28(18.62)	2.61(0.07)
(b) In the presence of BSA			
Synaptosomes	10	61.70(4.46)	
10mM glucose	5	53.35(5.38)	0.93(0.06)
0.5μmoles ADP	4	47.56(1.49)	0.99(0.01)
100μM DNP	5	111.09(4.58)	2.31(0.03)

Measured at 30°C in Krebs phosphate medium. Values for respiratory activity are expressed as μmoles O_2/hr/100mg protein; Mean (S.E.M.).

These results show that DNP in the absence of BSA significantly increases ($P<0.01$) the rate of oxygen uptake when compared to the rate in the presence of BSA. This suggests that BSA is in some way preventing the full uncoupling process.

Bradford (1969) showed that the presence of glucose increases the rate of oxygen uptake by synaptosomes. The method used here in the experiments described so far measures oxygen uptake on the addition of various substances over a relatively short time period (< 5 min) although the cycle of additions will take approximately 20 min to complete. The addition of glucose for longer time periods as used by Bradford (1969) may resolve this difference.

7. THE INFLUENCE OF VARYING OXYGEN CONCENTRATIONS ON SYNAPTOSOMAL OXYGEN UPTAKE - THE SEARCH FOR A CRITICAL, LIMITING OXYGEN CONCENTRATION

7.1. METHODS

7.1.1. Preparation of synaptosomes

Rat cerebral cortex synaptosomes were prepared by Methods 3, 4 and 5 as described in Section 3, based on Bradford et al. (1975). The differences will be described in the text.

7.1.2. Media

Krebs phosphate medium (Bradford et al., 1975).

Composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2.; $CaCl_2$, 0.75; $MgSO_4$, 1.3; Na_2HPO_4, 20 at pH 7.8.

7.1.3. Experimental design

(a) Normal oxygen tensions (P_{O_2}).

200µl samples of synaptosomes (∿3.36mg protein) resuspended in Krebs phosphate medium were added to the oxygen electrode chamber to give a final volume of 3ml. The medium within the oxygen electrode chamber was at 30°C and had been fully aerated by gassing with air that had been passed through a gas-wetter.

The synaptosomes in the closed chamber are allowed to consume all the available oxygen in solution and this rate is drawn out against time on a chart recorder. The results are usually expressed as µmoles O_2/hr/100mg protein and are plotted with respect to the oxygen concentration.

Krebs phosphate medium at 30°C, gassed with air will have a P_{O_2} = 158.8 mmHg (see Section 5.1.). This P_{O_2} will be termed "high" with reference to the experiments to be described.

The resuspended synaptosomal pellets are stored on ice until required and all variations in the composition of the medium are made before the addition of the synaptosomes to the closed oxygen electrode chamber.

(b) Reduced oxygen tensions

A gas mixer (see Section 5.2.) was used to vary the P_{O_2} in the medium before it was placed in the oxygen electrode chamber. Mixing air and N_2 proved successful for P_{O_2} values down to approximately 16mmHg.

For the "low" oxygen tension experiments, an initial P_{O_2} of 10mmHg was required and this was produced by direct gassing with N_2 into the medium in the oxygen electrode chamber. The P_{O_2} can be monitored on the chart recorder and the plunger placed in position when the predetermined value is reached. Increasing the scale expansion factor aids measurement from the chart recorder trace.

The following Table 7.1.3. provides a conversion between the expression of the oxygen content as P_{O_2} (mmHg) and oxygen concentration (μM). See also Section 5.1.

TABLE 7.1.3. Conversion table between O_2 tension and O_2 concentration in solutions at 30°C

$[O_2]$, µM	P_{O_2}, mmHg	$[O_2]$, µM	P_{O_2}, mmHg
1	0.72	8	5.75
2	1.44	9	6.47
3	2.16	10	7.19
4	2.88	11	7.91
5	3.60	12	8.63
6	4.31	13	9.35
7	5.03	14	10.07
		220	158.8

7.2. RESULTS

7.2.1. Oxygen uptake by synaptosomes placed in an air-saturated medium

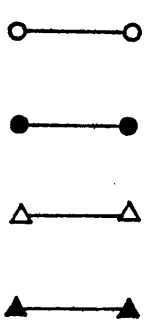
200µl samples of synaptosomes (∿3.36mg protein) were placed in air-saturated Krebs phosphate medium at 30°C containing the following: 10mM glucose; 10mg BSA (3.33mg/ml), or 10mM glucose plus 10mg BSA. Final volume 3ml. For results see Figure 7.2.1.(a).

BSA alone had no significant effect on synaptosomal oxygen uptake at high oxygen tensions whereas both glucose and glucose plus BSA showed a significant stimulation in the rate of oxygen uptake ($P<0.01$ and $P<0.05$ respectively).

It seems likely that the stimulation produced by glucose plus BSA is due principally to the presence of the glucose.

FIGURES 7.2.1.(a) and (b). OXYGEN UPTAKE BY SYNAPTOSOMES PLACED IN AN AIR-SATURATED MEDIUM

KEY



P_2B (synaptosomes)

P_2B + glucose (10mM)

P_2B + BSA (3.33mg/ml)

P_2B + glucose + BSA

Measured at 30°C in air-saturated Krebs phosphate medium. The ratio of tissue (mg) to BSA (mg) was 3.36 : 10.

Fig. 7.2.1.(a) shows error bars (S.E.M.) on the first determinations only as representative of the remaining values for the rate of synaptosomal O_2 uptake as the O_2 concentration falls. (n = 4-5).

Fig. 7.2.1.(b) is a cumulative diagram of synaptosomal O_2 uptake. Values given are Mean ± S.E.M. (n = 3-5).

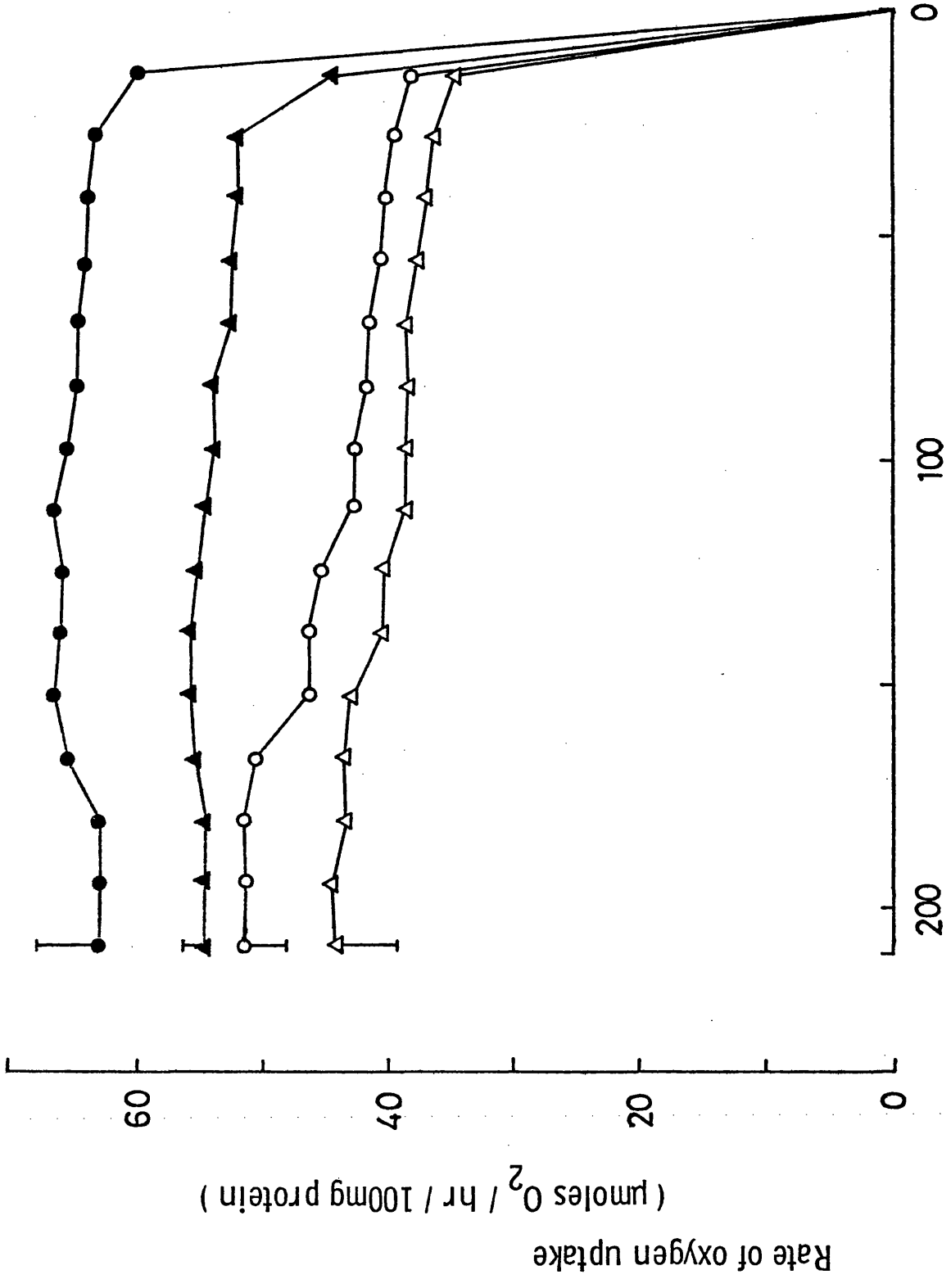
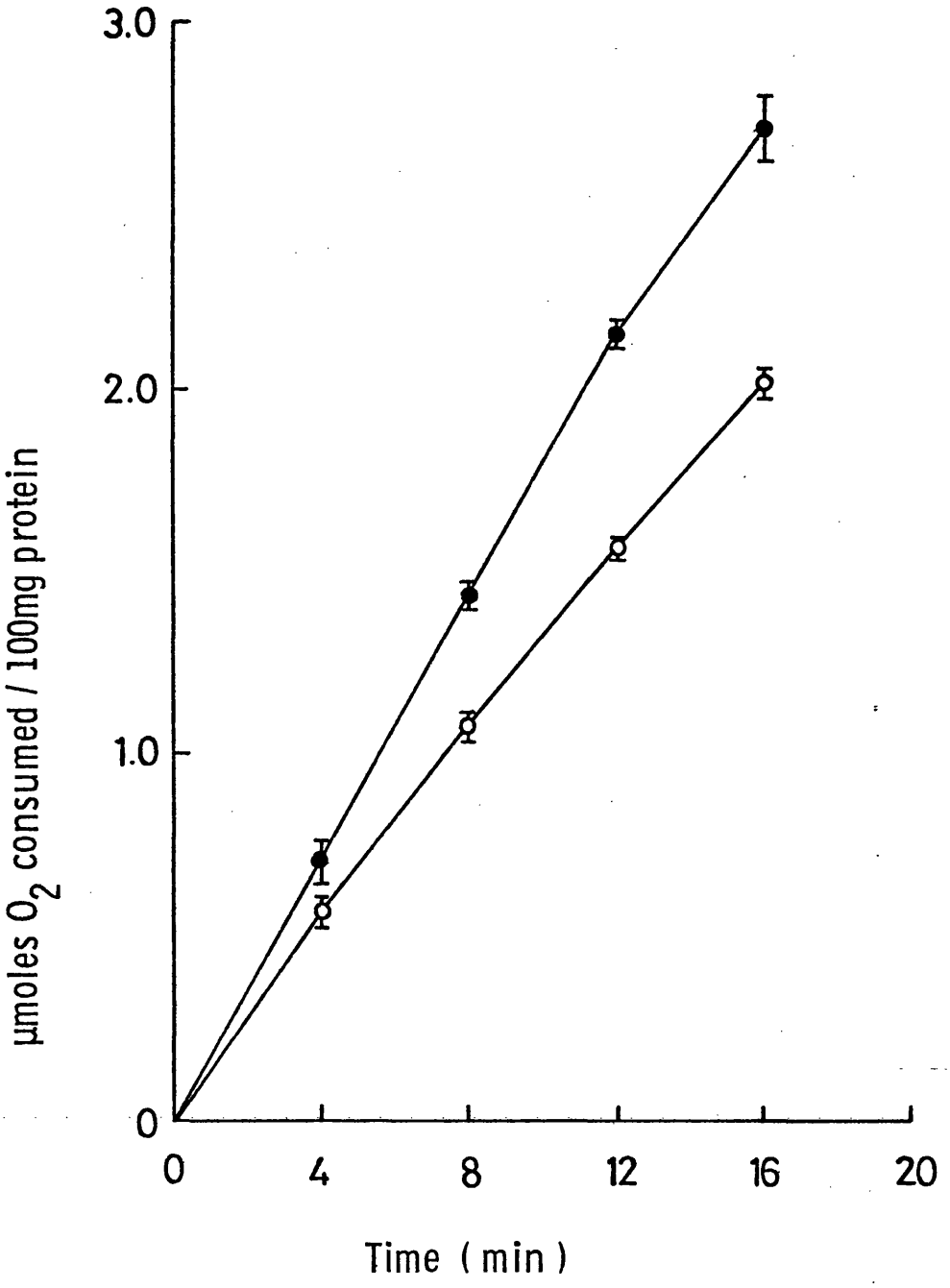

Rate of oxygen uptake
(μmoles O_2 / hr / 100mg protein)
0
20
40
60
0
100
200
Oxygen concentration (μM)

FIGURE 7.2.1. (a).

FIGURE 7.2.1. (b).


3.0
2.0
1.0
0
µmoles O_2 consumed / 100mg protein
0
4
8
12
16
20
Time (min)

Consideration of the results with and without glucose between P_{O_2} 150-20mmHg confirms, by analysis of variance for testing regression goodness-of-fit (Clark, 1969), that the glucose rate is horizontal whereas the Krebs line is not (Glucose: $F = 222.74_{(1,12)}$; Krebs: $F = 0.128_{(1,12)}$). The representation of the glucose rate having zero gradient infers that the presence of glucose maintains the rate of oxygen uptake by synaptosomes at a more constant level than in its absence.

This is confirmed by two other observations: (1) there is a significant difference ($P<0.05$) between the rate of oxygen uptake at P_{O_2} 150mmHg and 20mmHg in the absence of glucose. With 10mM glucose there is no significant difference in the rates at P_{O_2} 150 and 20mmHg. (2) Presentation of the results in a different manner visualizes this difference more clearly, see Figure 7.2.1.(b).

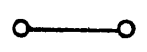
7.2.2. The effect on synaptosomal oxygen uptake of varying the concentration of oxygen in the medium

From the results represented in Figure 7.2.1.(a), it seemed likely that a critical oxygen concentration in terms of oxygen uptake by synaptosomes would be found around a concentration below 30μM. In order to find a closer estimate for a critical oxygen concentration, synaptosomes were incubated at 30°C in media of various reduced initial P_{O_2} values, again containing 10mM glucose and BSA (3.33mg/ml). 100μl samples of synaptosomes (∿ 1.68mg protein) were used throughout. For results see Figures 7.2.2.(a) - (e).

No error bars have been drawn on Figures 7.2.2.(a) - (d) because of the low sample numbers involved (n = 1-3). However there does appear to be a fairly constant trend throughout with the rate of oxygen uptake increasing in the following order: Krebs < glucose < BSA < glucose plus BSA.

FIGURES 7.2.2.(a)-(e). THE EFFECT ON SYNAPTOSOMAL OXYGEN UPTAKE OF VARYING THE INITIAL CONCENTRATION OF OXYGEN IN THE MEDIUM

KEY


P_2B (synaptosomes)


P_2B + glucose (10mM)


P_2B + BSA (3.33mg/ml)


P_2B + glucose + BSA

Measured at 30°C in Krebs phosphate medium. The ratio of tissue (mg) to BSA (mg) was 1.68 : 10.

No error bars have been given on Figs. 7.2.2.(a)-(d) because of the low sample numbers (n = 1-3).

Fig. 7.2.2.(e) shows error bars (S.E.M.) on the first determinations only as representative of the remaining values for the rate of synaptosomal O_2 uptake as the O_2 concentration falls. (n = 4-7).

FIGURE 7.2.2. (a).

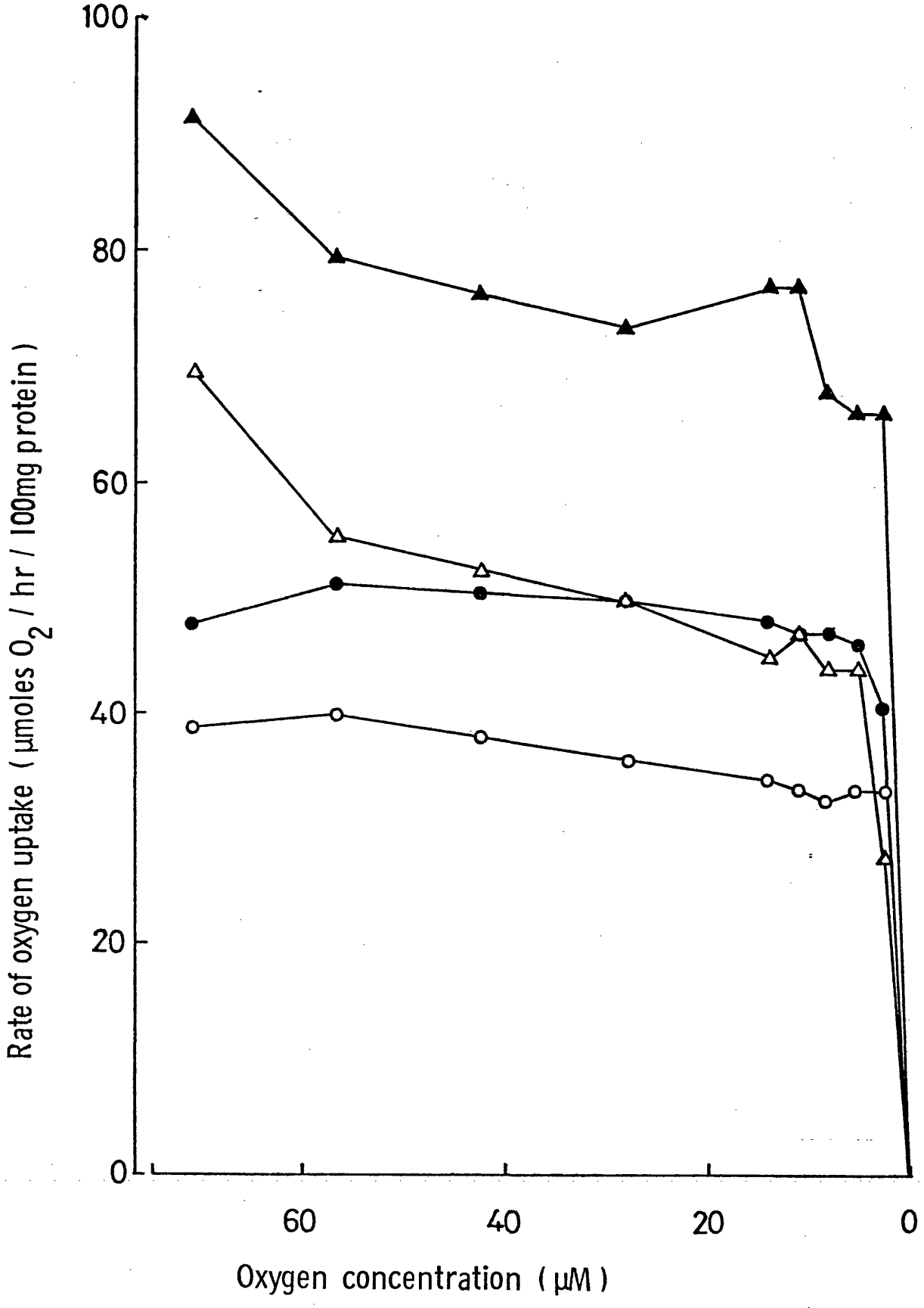

100
80
60
40
20
0
Rate of oxygen uptake (µmoles O_2 / hr / 100mg protein)
60
40
20
0
Oxygen concentration (µM)

FIGURE 7.2.2. (b).

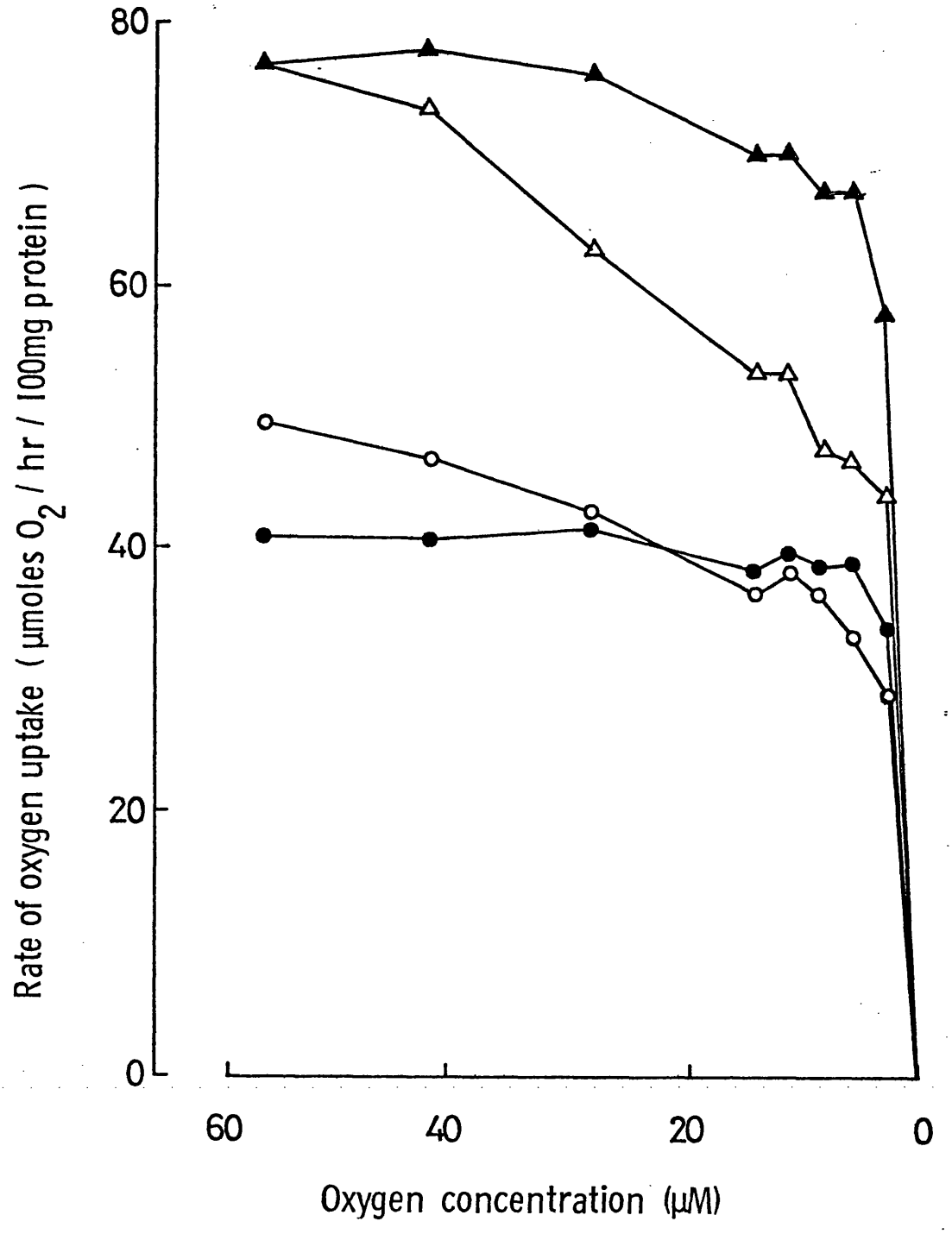

80
60
40
20
0
Rate of oxygen uptake (µmoles O_2 / hr / 100mg protein)
60
40
20
0
Oxygen concentration (µM)

FIGURE 7.2.2. (c).

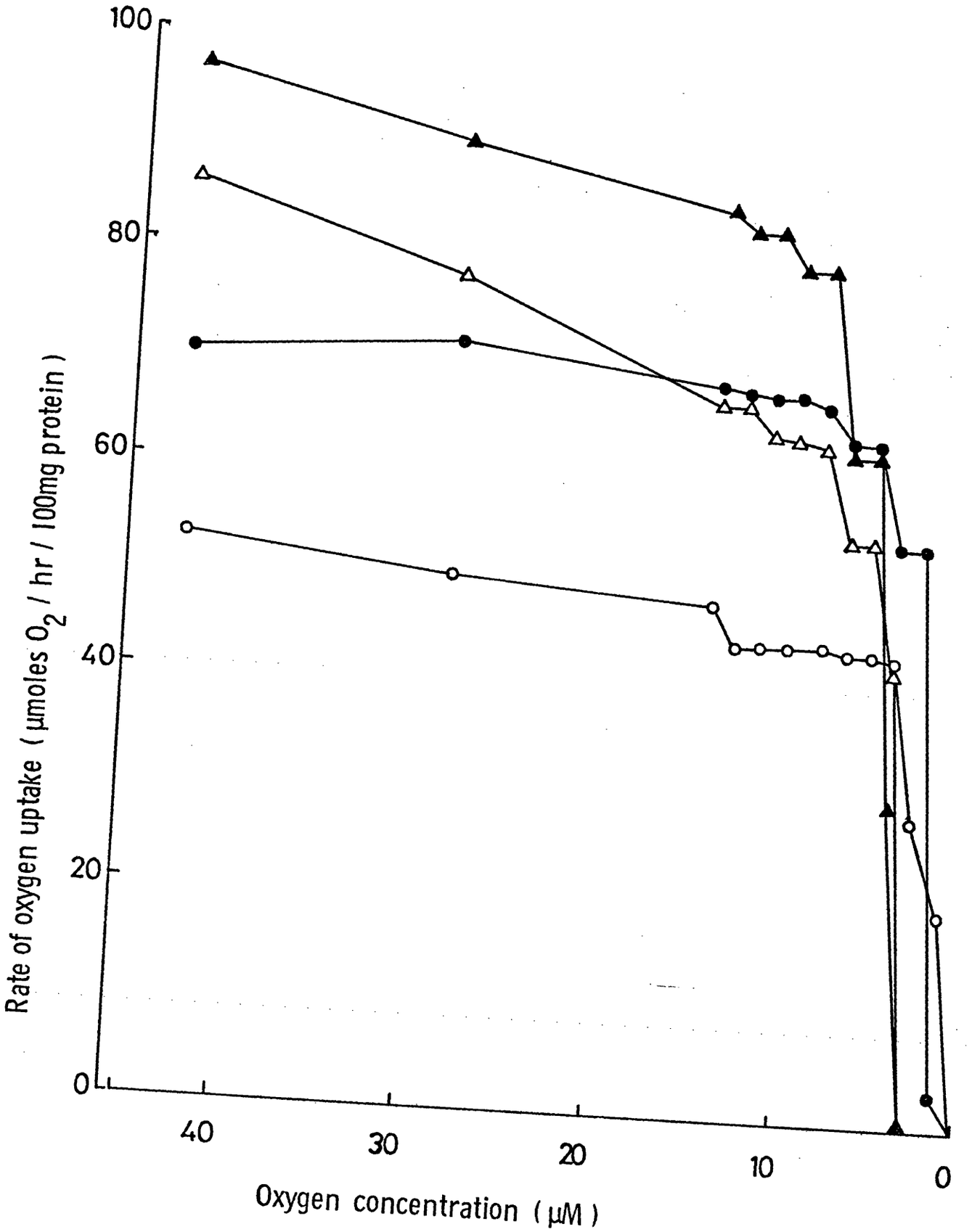

100
80
60
40
20
0
Rate of oxygen uptake (µmoles O_2 / hr / 100mg protein)
40
30
20
10
0
Oxygen concentration (µM)

FIGURE 7.2.2. (d).

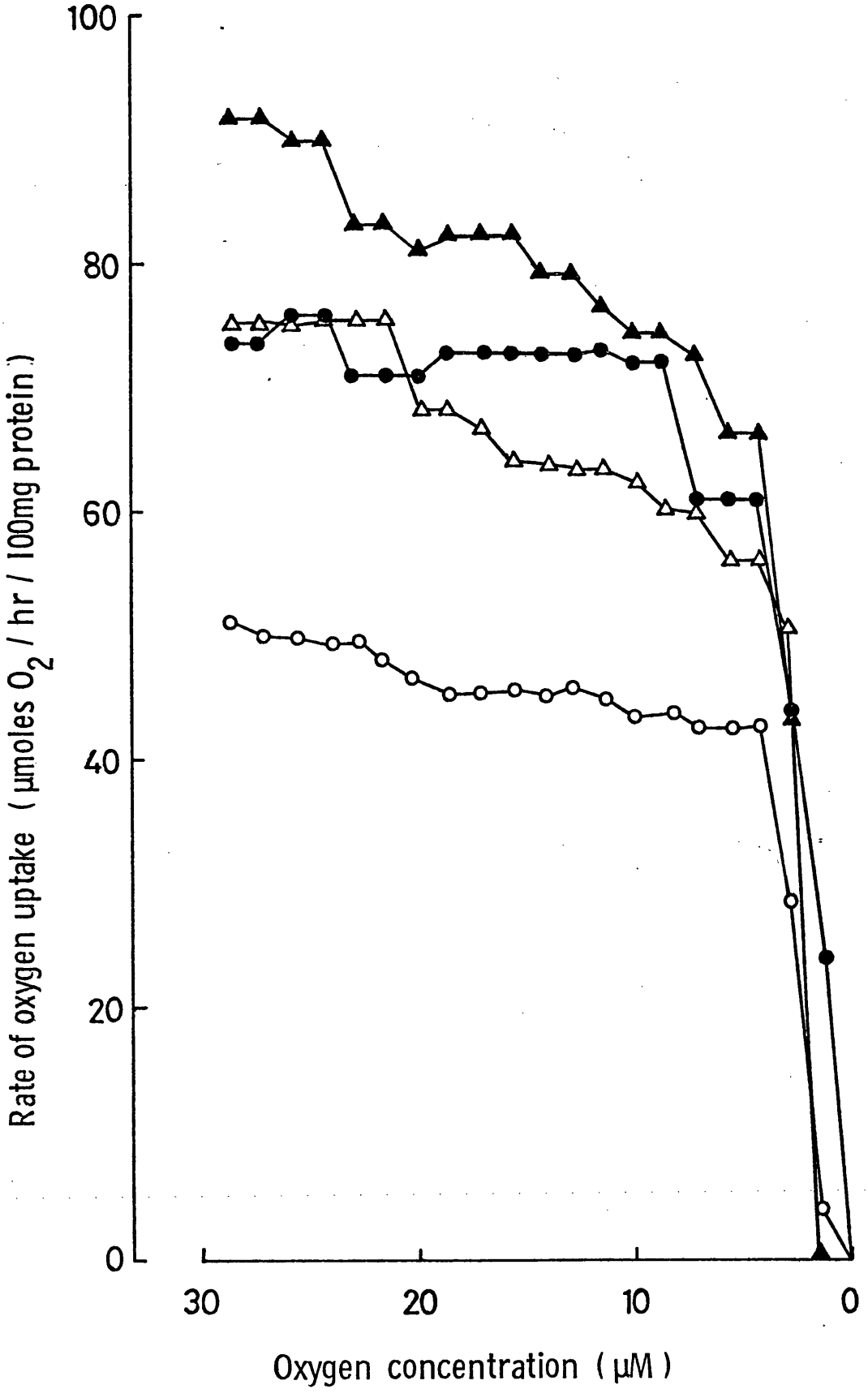

100
80
60
40
20
0
30
20
10
0
Rate of oxygen uptake (µmoles O_2 / hr / 100mg protein)
Oxygen concentration (µM)

FIGURE 7.2.2. (e).

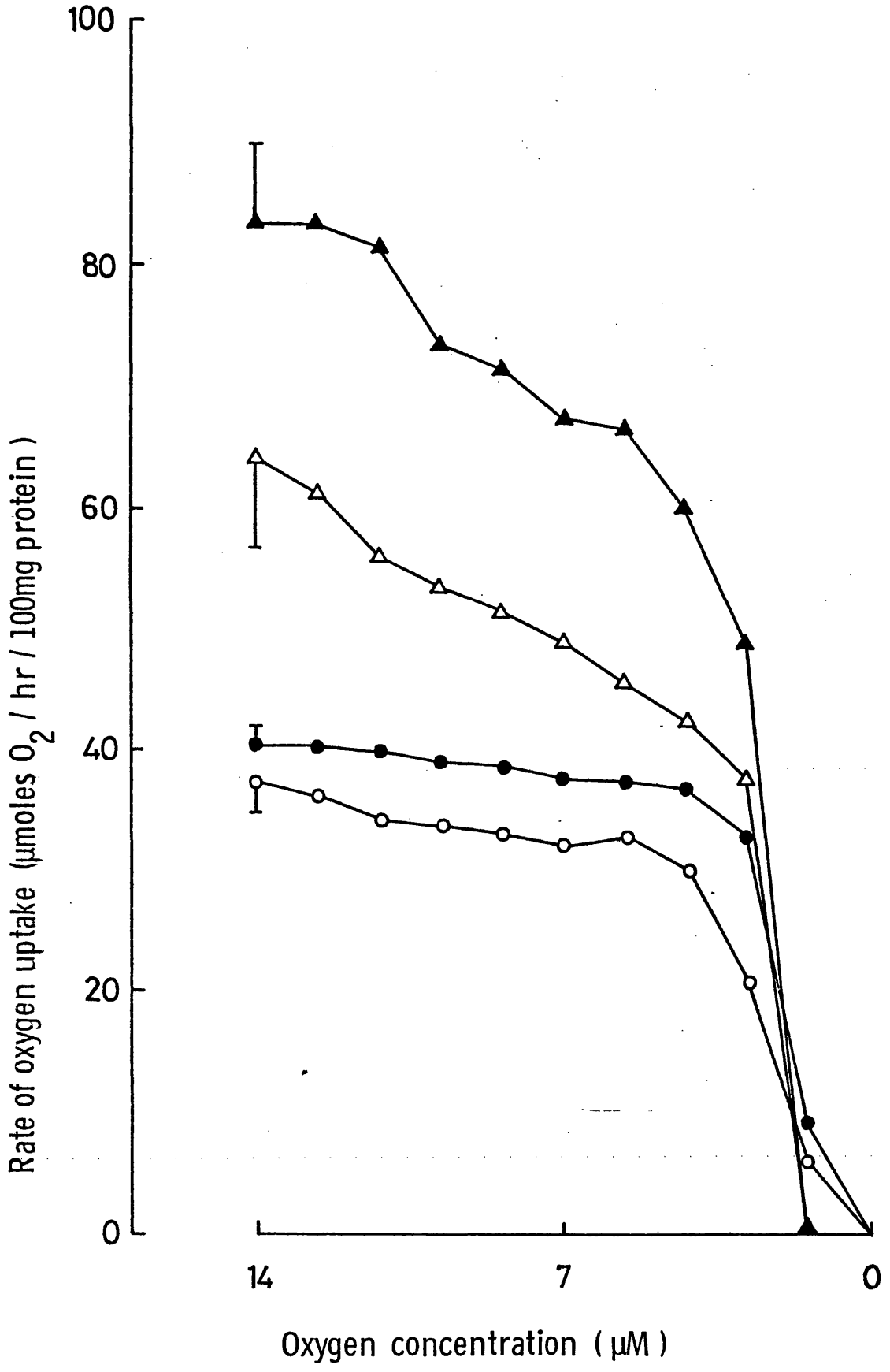

100
80
60
40
20
0
Rate of oxygen uptake (µmoles O_2 / hr / 100mg protein)
14
7
0
Oxygen concentration (µM)

Figure 7.2.2.(e) shows that the glucose stimulation becomes significant ($P<0.05$) at an oxygen concentration of 4-5μM with both BSA and glucose plus BSA producing a more significant stimulatory response ($P<0.01$ and $P<0.001$) respectively.

7.2.3. Combination of results from experiments starting at high and low oxygen concentrations

By combining all the results from Sections 7.2.1. and 7.2.2. a strange picture emerges with reference to the presence of BSA at oxygen concentrations of 70μM and less, see Figure 7.2.3.(i).

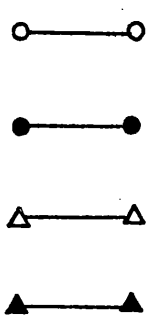
10mM glucose increases the rate of oxygen uptake by synaptosomes over the whole range of oxygen concentrations whereas BSA alone only becomes significant at an oxygen concentration below 30μM. The ratio of tissue to BSA may be implicated here since the experiments started in air-saturated media have a ratio of 200μl (∿3.4mg protein) to 10mg BSA in contrast to the low P_{O_2} experiments with ratios of 100μl (∿ 1.7mg protein) to 10mg BSA. 10mM glucose would seem to be in excess as no marked differences are observed.

Expansion of the results from 14μM oxygen show that glucose and BSA may be acting by different mechanisms since the addition of both together suggests an additive response, see Figure 7.2.3.(ii).

The notion of a critical oxygen concentration for synaptosomal oxygen uptakes does appear compatible with these results. At any oxygen concentration below approximately 4μM, oxygen itself seems to become limiting in terms of the overall process of oxygen consumption by synaptosomes, a process that appears to be relatively independent of the oxygen concentration above 4μM to 220μM.

FIGURES 7.2.3.(i) and (ii). COMBINED RESULTS OF SYNAPTOSOMAL OXYGEN UPTAKE STARTING AT DIFFERENT OXYGEN CONCENTRATIONS

KEY



Measured at 30°C in Krebs phosphate medium. The ratio of tissue (mg) to BSA (mg) was 3.4 : 10 for O_2 concentrations greater than and 1.7 : 10 for O_2 concentrations below approx. 70μM.

Representative error bars (S.E.M.) are shown. For Fig. 7.2.3.(i), n = 5-20 and for Fig. 7.2.3.(ii) which shows in more detail the rates of synaptosomal O_2 uptake at low O_2 concentrations, n = 19-21.

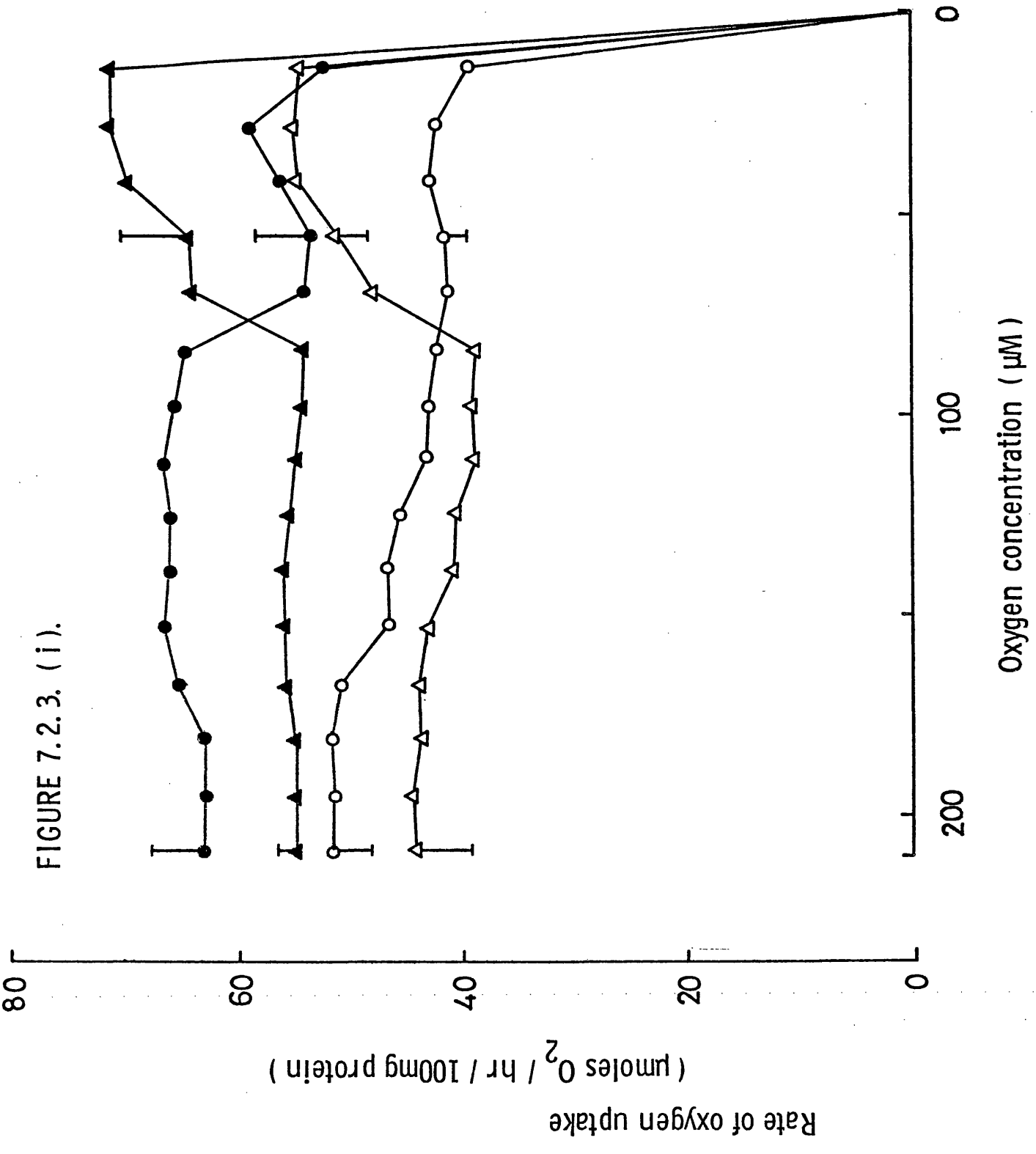
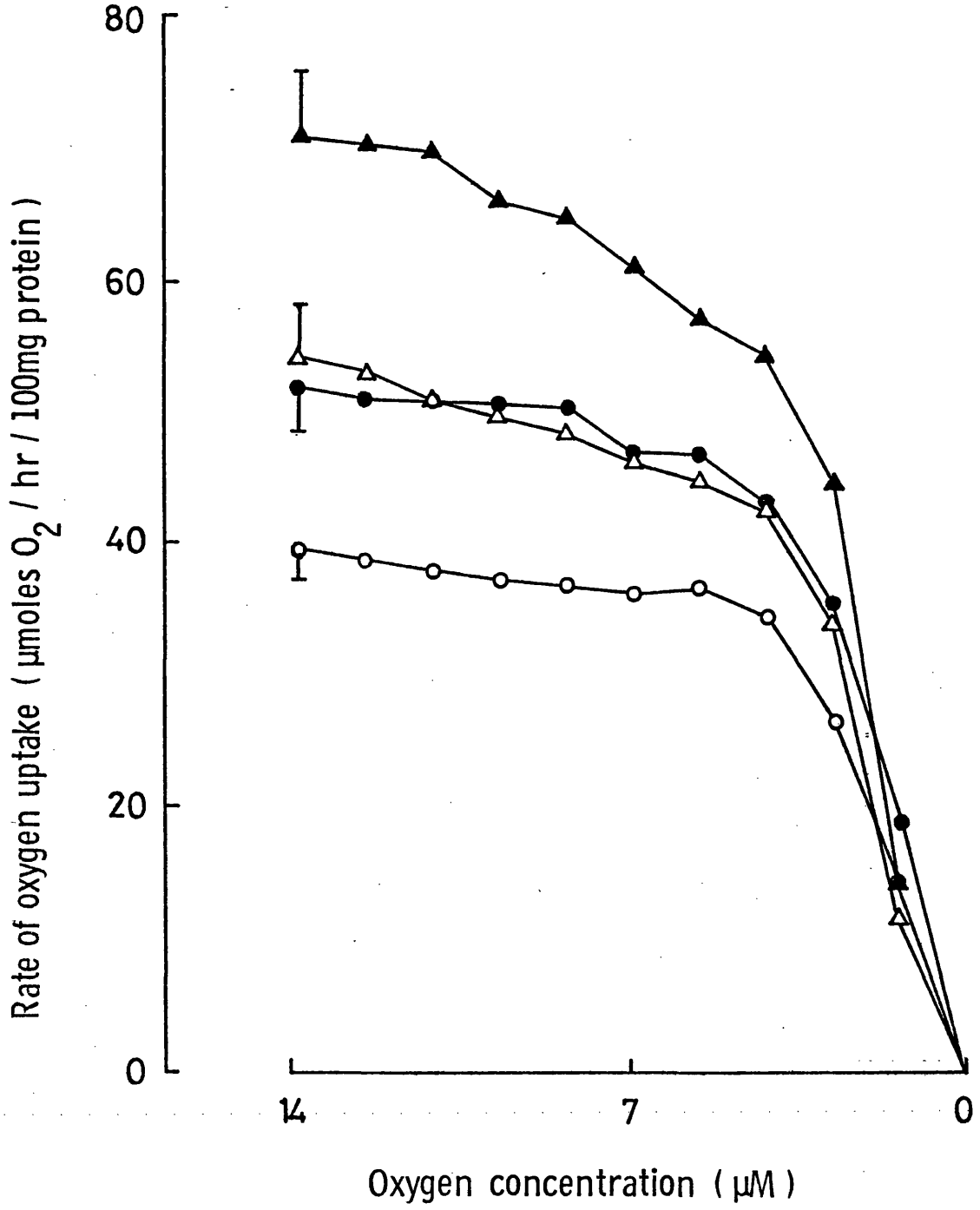

Rate of oxygen uptake
(µmoles O_2 / hr / 100mg protein)
80
60
40
20
0
0
100
200
Oxygen concentration (µM)

FIGURE 7.2.3. (i).

FIGURE 7.2.3. (ii).



7.3.1. The effect of different homogenization conditions on synaptosomal oxygen uptake

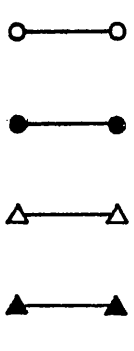
Unforseen circumstances forced me to look at the effect of using different combinations of pestles and tubes for the initial homogenization procedure. In all the experiments reported so far, the pestle and tube were of the following specifications: (1) Clearance 0.25mm; glass-reinforced teflon pestle, length 3cm (shearing surface). In this section, results were obtained with the following pairs: (2) Clearance 0.25mm; teflon pestle, length 1.3cm, (3) clearance 0.50mm; glass-reinforced teflon pestle, length 3cm.

Using air-saturated medium, synaptosomal oxygen uptake was measured at 30°C in Krebs phosphate medium containing glucose and/or BSA, see Figure 7.3.1.(i) and (ii). Error bars have not been included due to low sample numbers (n = 1-7).

Although the results are quantitatively different, they all show the same qualitative responses, i.e. BSA is without effect and glucose increases oxygen uptake. For a comparison of rates in Krebs phosphate medium along see Table 7.3.1.

FIGURES 7.3.1.(i) and (ii). THE EFFECT OF DIFFERENT HOMOGENIZATION CONDITIONS ON SYNAPTOSOMAL OXYGEN UPTAKE

KEY



Measured at 30°C in air-saturated Krebs phosphate medium. The ratio of tissue (mg) to BSA (mg) was 3.4 : 10. No error bars have been shown due to low sample numbers (n = 1-7).

Fig. 7.3.1.(i) shows the results obtained with pestle and tube pair (2), and Fig. 7.3.1.(ii) shows the results using pair (3) as described in Section 7.3.

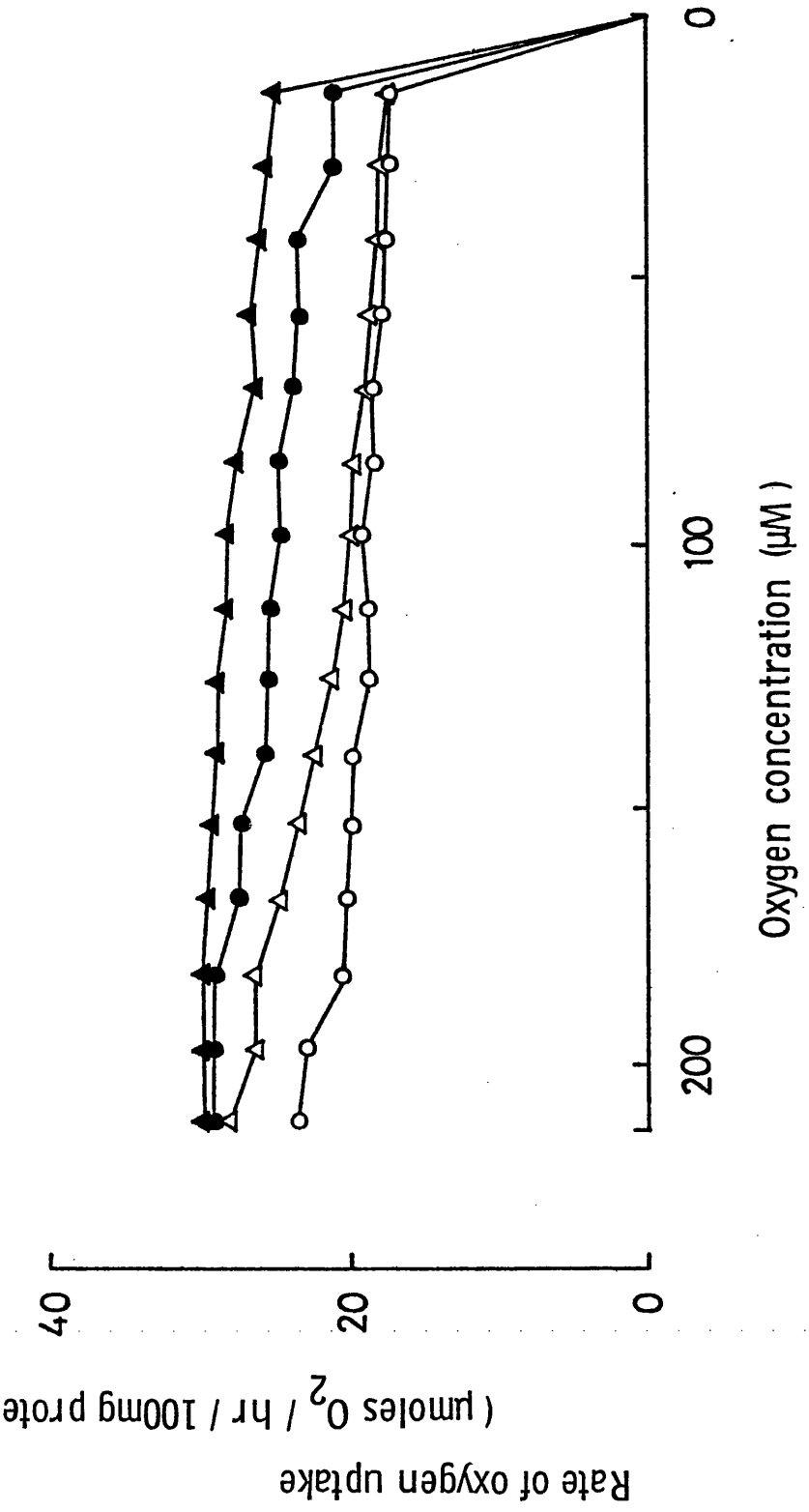

Rate of oxygen uptake
(µmoles O_2 / hr / 100mg protein)
40
20
0
0
100
200
Oxygen concentration (µM)

FIGURE 7.3.1. (I).

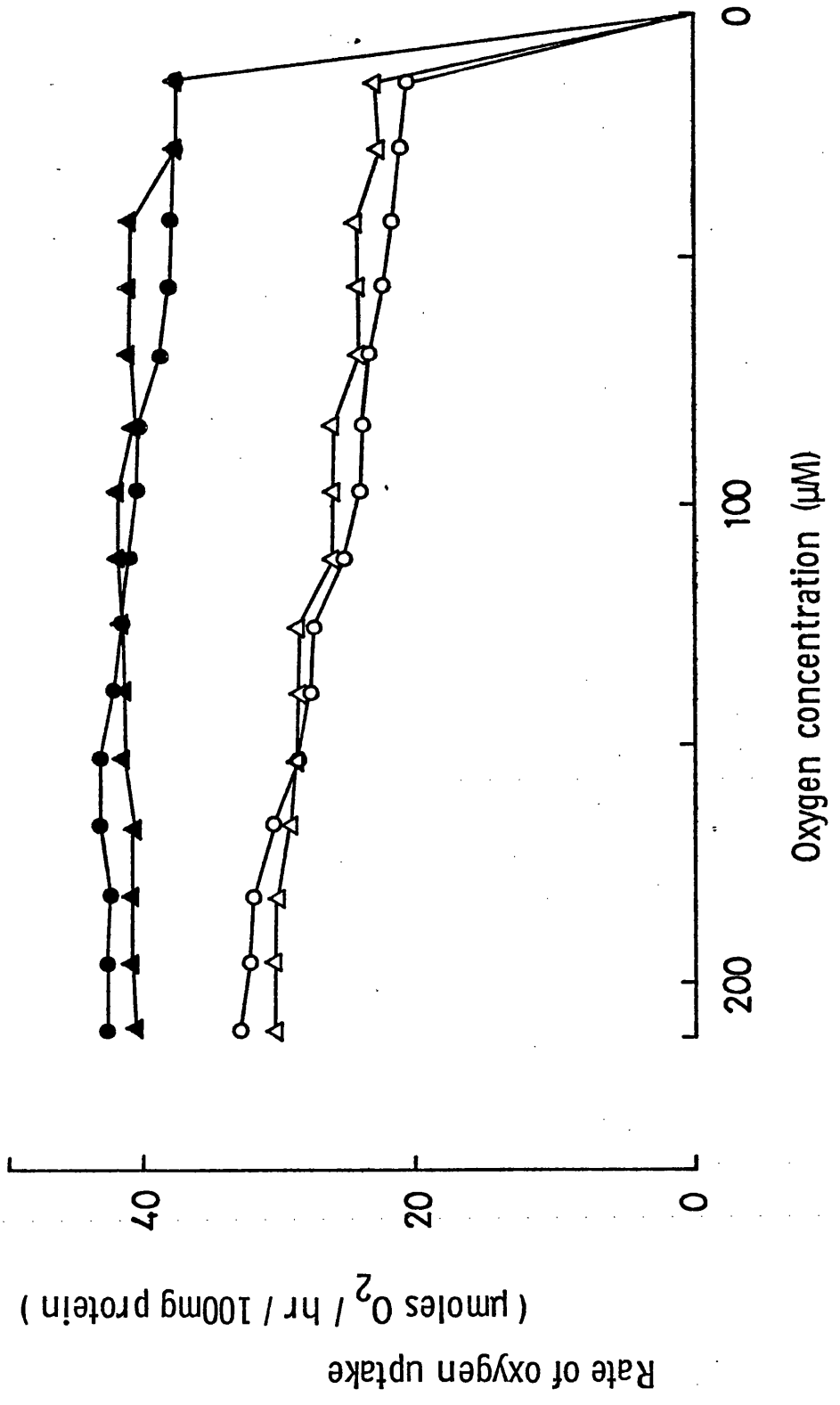


FIGURE 7.3.1. (ii).

TABLE 7.3.1. Comparison of the rate of oxygen uptake at 220μM O_2 and the protein concentration of the resuspended synaptosomal pellet using different homogenization procedures

Procedure	Rate of O_2 uptake	Protein (mg/ml)
1	51.5(3.5) n = 5	16.76(0.50) n = 32
2	24 n = 2	13.50(0.35) n = 45
3	33 n = 4	17.50(0.83) n = 21

Rates measured at 30°C in Krebs phosphate medium. Values for respiratory activity are expressed as μmoles O_2/hr/100mg protein; Mean (S.E.M.). Explanation of procedure numbers in text.

Procedures 1 and 2 are identical except that the length of the pestle in 1 is about twice the length of the pestle in 2. Judging from the results in Table 7.3.1., the respiratory activity of the synaptosomes similarly indicates a 2 : 1 relationship between procedures 1 and 2.

The use of a larger clearance in 3 also reduces respiratory activity suggesting that larger clearances produce a less efficient conversion of intact nerve terminals to synaptosomes. These results taken together stress the necessity of using identical conditions throughout the course of an experimental series in order that any preparative errors can be minimized, thus making results comparable.

It would seem that the original pair of pestle and mortar (1) was required to produce optimal respiratory activity of synaptosomes, giving values comparable with Bradford (1969) for glucose-supported oxygen uptake. The influence of relatively small changes in homogenization conditions has been discussed by Whittaker & Dowe (1965).

7.3.2. The characteristic response of synaptosomes to the presence of glucose and bovine serum albumin.

Figure 7.3.2. combines the results of Sections 7.2.1., 7.3.1.(i) and (ii). The results are expressed as a percentage of the control rate, taken as the rate of oxygen uptake in Krebs phosphate medium alone.

Separate values are obtained under the three conditions of homogenization used and have been combined together. Emerging from this is support for the idea that at high oxygen tensions, BSA at the concentration used has no effect whereas glucose is capable of stimulating the rate of oxygen uptake by synaptosomes. The increase seen by the presence of glucose plus BSA again seems due essentially to the presence of the glucose.

When expressed as a percentage of the control rate, the glucose value increases as the oxygen concentration falls. This being due to the steady decline in the control rate as compared to the relatively stable rate produced by the presence of 10mM glucose (and glucose plus BSA).

FIGURE 7.3.2. COMBINED RESULTS OF THE EFFECTS ON SYNAPTOSOMAL OXYGEN UPTAKE OF DIFFERENT HOMOGENIZATION CONDITIONS

KEY

●——● P_2B (synaptosomes) + glucose (10mM)

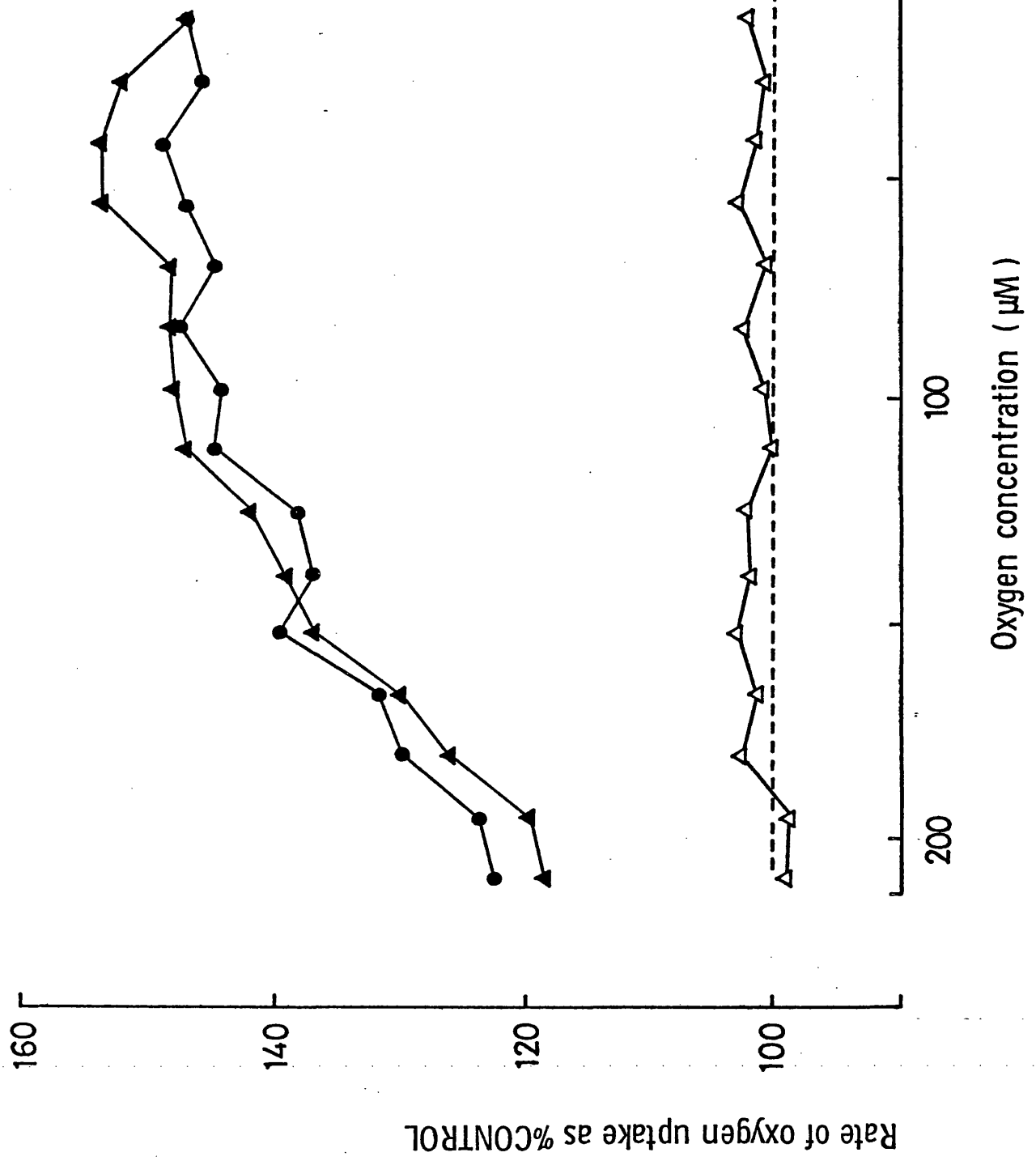
△——△ P_2B + BSA (3.33mg/ml)

▲——▲ P_2B + glucose + BSA

Measured at 30°C in air-saturated Krebs phosphate medium.

Fig. 7.3.2. combines the results expressed in Figs. 7.2.1.(a), 7.3.1.(i) and 7.3.1.(ii) in order to highlight the consistent effects of glucose and BSA on synaptosomal O_2 consumption regardless of the initial homogenization conditions.

FIGURE 7.3.2.


160
140
120
100
Rate of oxygen uptake as %CONTROL
CONTROL
0
100
200
Oxygen concentration (μM)

8. WHAT IS THE RELATIONSHIP BETWEEN THE PROCESS OF O_2 UPTAKE BY SYNAPTOSOMES PLACED IN MEDIA OF HIGH AND LOW O_2 CONCENTRATIONS?

8.1. METHODS

8.1.1. Preparation of synaptosomes

Rat cerebral cortex synaptosomes were prepared by Method 5 as described in Section 3, based on Bradford et al. (1975).

8.1.2. Media

Krebs phosphate medium (Bradford et al., 1975). Composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$ 0.75; $MgSO_4$, 1.3; Na_2HPO_4, 20 at pH 7.8.

8.1.3. Experimental design

High and low P_{O_2} experiments were performed as described in Section 7.1.3. Due to the lower activity of the synaptosomes when prepared with the relatively short pestle (see Table 7.3.1.), it was found necessary to use larger samples of tissue for the high P_{O_2} experiments. High P_{O_2} experiments used 400µl samples compared to 100µl samples for low P_{O_2} experiments. The final volume in the oxygen electrode chamber was always 3ml. 100µl of resuspended synaptosomal pellet = 1.46mg protein (S.E.M., 0.12; n = 5).

The pre-incubation experiments were performed as follows:

All control experiments used synaptosomes which were kept on ice until required. The synaptosomal pellet was resuspended in 200µl Krebs phosphate medium and samples introduced to the oxygen electrode chamber containing (final volume 3ml), 10mM glucose, 10mg BSA (3.33mg/ml) or glucose plus BSA.

Pre-incubation was performed by placing the resuspended pellet, still in the 10 x 10ml centrifuge tube used in the last stage of the preparation, in a water bath at 30°C. Two water baths were used,

(i) a non-shaking and (ii) a shaking water bath. For the pre-incubation experiments, the synaptosome pellets were resuspended in (1) Krebs phosphate medium, (2) Krebs plus 10mM glucose, or (3) Krebs plus BSA (3.33mg/ml).

When 100µl samples of (2) and (3) were placed in the oxygen electrode chamber containing Krebs phosphate medium, the final concentration of glucose became 0.27mM and the concentration of BSA was 0.09mg/ml.

TABLE 8.1.3. Explanation of abbreviations used in the pre-incubation experiments

Composition of resuspending medium	Composition of medium in O_2 electrode chamber			
	Krebs	10mM glucose	BSA 3.33mg/ml	Glucose + BSA
Krebs	Krebs (Control)	G(c)	B(c)	G + B (c)
10mM Glucose	G(p)	G(p + c)		
BSA (3.33mg/ml)	B(p)		B(p + c)	
Glucose + BSA	G + B (p)			G + B (p + c)

All solutions are made up in Krebs phosphate medium

8.2. RESULTS

8.2.1. Characterisation of responses to high and low oxygen tensions by synaptosomes

The use of a shorter pestle for the initial homogenization of the rat brain involved the production of synaptosomes of reduced respiratory activity (see Table 7.3.1.). For this reason it was necessary to repeat the experiments as described in 7.2.1. and 7.2.2.

(i) Oxygen uptake by synaptosomes placed in air-saturated medium.

400µl samples of synaptosomes (∿ 5.8mg protein) were placed in the oxygen electrode chamber containing air-saturated Krebs phosphate medium at 30°C (final volume 3ml). The results are shown in Figure 8.2.1.(i) and correspond well with results previously obtained (see 7.2.1.).

Analysis of results at an oxygen concentration of 100µM shows a significant increase ($P<0.01$) in synaptosomal oxygen uptake in the presence of 10mM glucose.

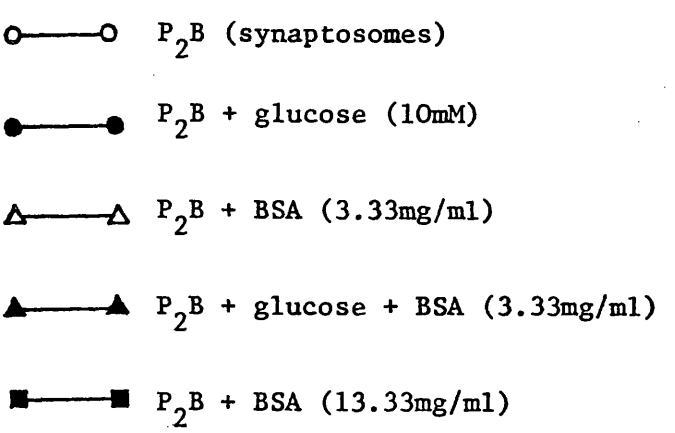
Two concentrations of BSA were used: 3.33mg/ml and 13.33mg/ml. This represents a tissue to BSA ratio of 5.8mg synaptosomal protein to 10mg BSA and 5.8mg synaptosomal protein to 40mg BSA (1.45 : 10). A ratio of 5.8 : 10 (tissue : BSA) produced a significant decrease in the rate of oxygen uptake ($P<0.001$) compared to a ratio of 1.45 : 10 which significantly increased oxygen uptake by synaptosomes ($P<0.05$).

(ii) Oxygen uptake by synaptosomes placed in media of low oxygen tension.

100µl samples of synaptosomes (∿ 1.46mg protein) were added to the oxygen electrode chamber containing 2.9ml media of reduced oxygen

FIGURES 8.2.1.(i) and (ii). THE CHARACTERISTIC RESPONSE OF SYNAPTOSOMAL OXYGEN CONSUMPTION TO GLUCOSE AND BOVINE SERUM ALBUMIN IN MEDIA OF HIGH AND LOW OXYGEN CONCENTRATION

KEY



Measured at 30°C in Krebs phosphate medium. In Fig. 8.2.1.(i) synaptosomes were placed in air-saturated medium and here the tissue (mg) to BSA (mg) ratio was 5.8 : 10 and 1.70 : 10 for the low and high concentrations of BSA respectively.

In Fig. 8.2.1.(ii) the medium was initially low in O_2 content and the tissue (mg) to BSA (mg) ratio was 1.45 : 10.

Representative error bars (S.E.M.) are shown, (n = 4-7).

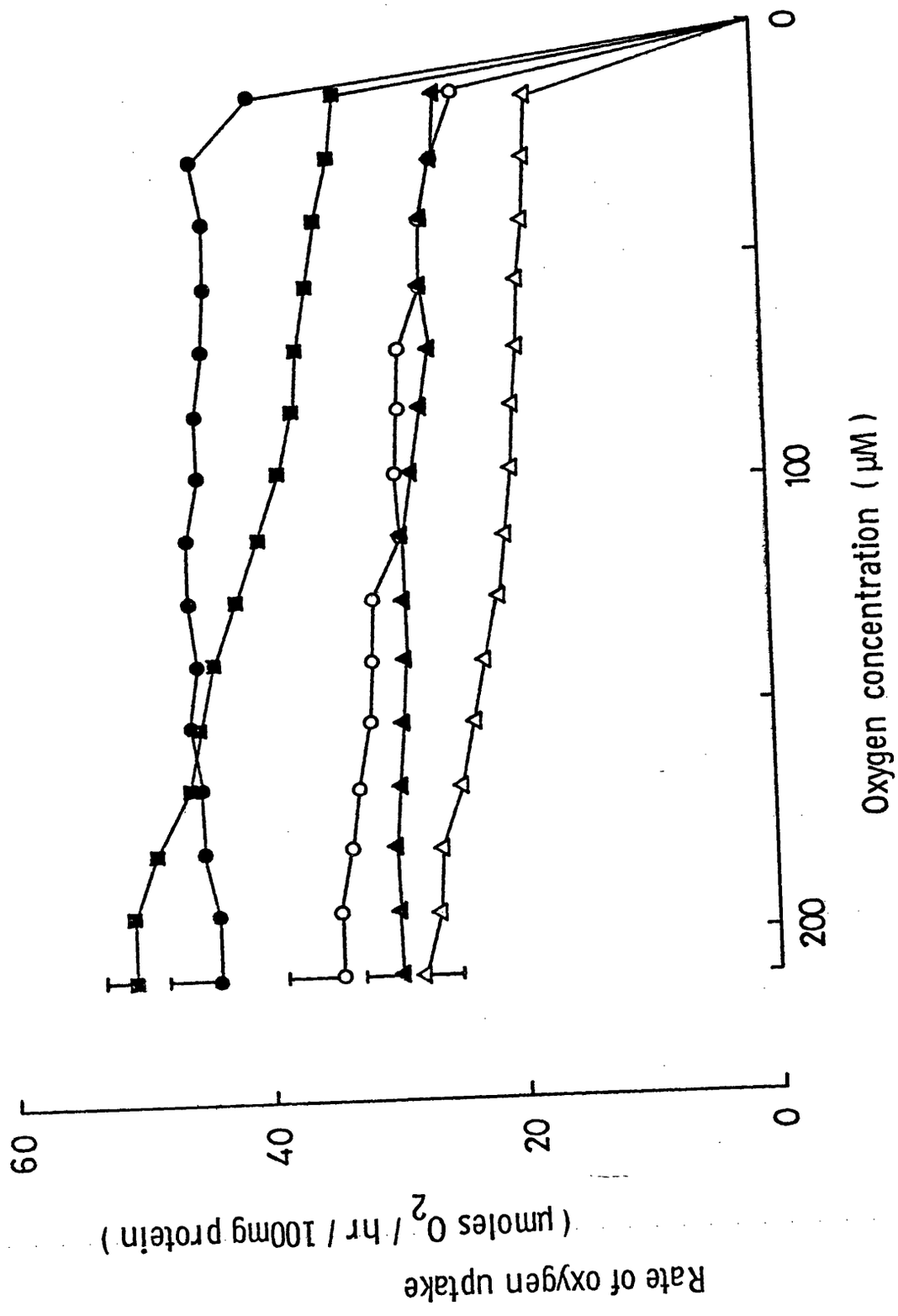
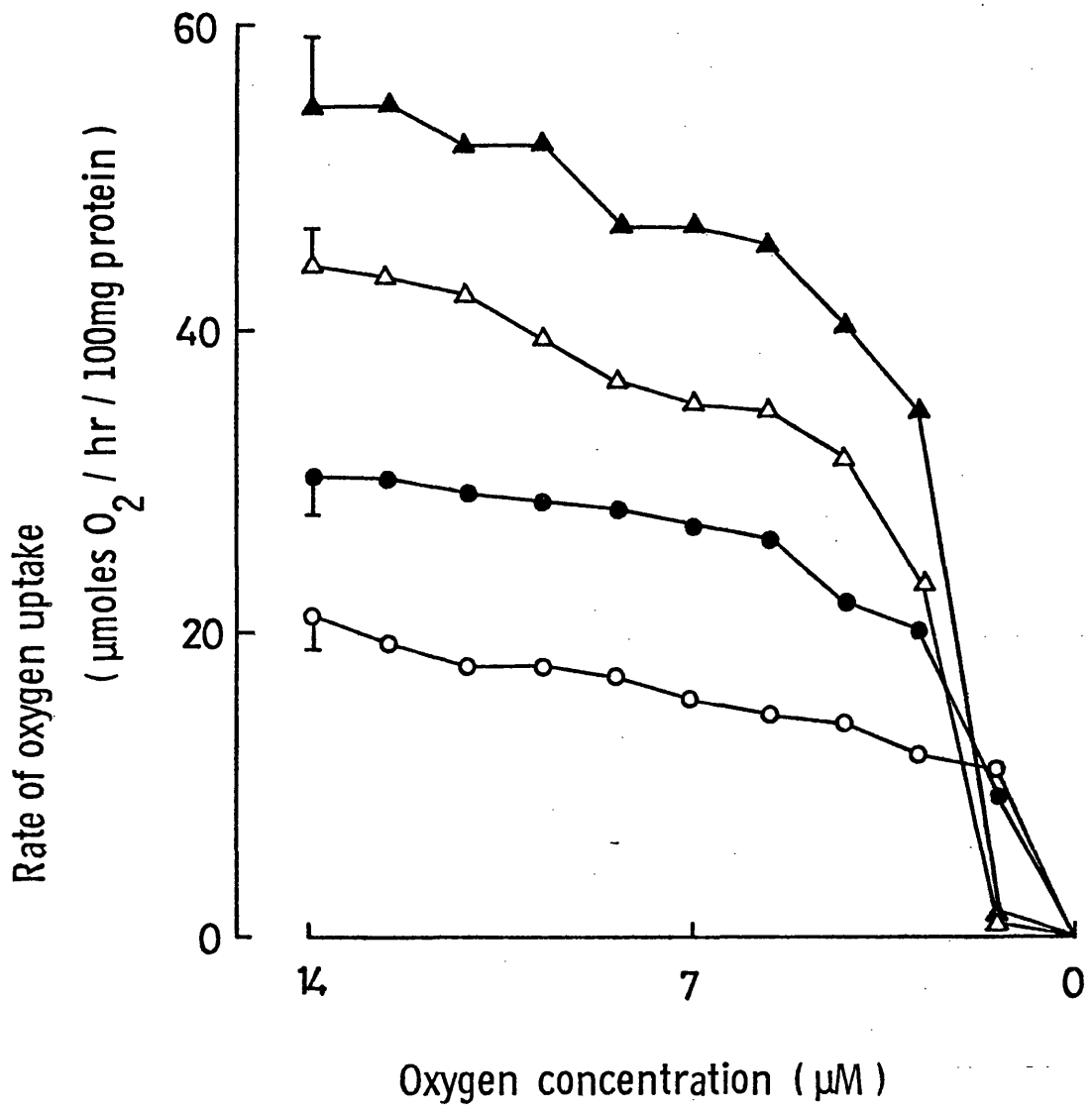

Rate of oxygen uptake
(μmoles O_2 / hr / 100mg protein)
60
40
20
0
0
100
200
Oxygen concentration (μM)

FIGURE 8.2.1. (i).

FIGURE 8.2.1. (ii).



concentration (14μM) at 30°C. The results are shown in Figure 8.2.1.(ii). As observed previously (see Section 7.2.2.), the presence of 10mM glucose significantly increased ($P<0.01$) oxygen uptake by synaptosomes at low P_{O_2} values.

Similarly addition of 10mg BSA, and glucose plus BSA, markedly increased the rate of oxygen uptake ($P<0.001$). The rate with glucose plus BSA again seemed to be an addition of the individual rates.

These results also compare well with those detailed in Section 7.2.3. suggesting the possibility of a critical oxygen concentration at or below 4μM.

8.2.2. A comparison of the initial rates of oxygen uptake at high and low oxygen tensions

On first sight, the impression gained from Figures 8.2.1.(i) and (ii) is that they differ principally with respect to the presence of BSA. However this might well be explained by consideration of the ratio of tissue (mg) to BSA (mg) as suggested in Section 8.2.1.(i).

In the high oxygen experiments we have a ratio of 5.8 : 10 (tissue : BSA) causing a decrease, and a ratio of 1.45 : 10 causing an increase, in oxygen uptake by synaptosomes. At low oxygen tensions, a ratio of 1.45 : 10 produced an even more highly significant increase in oxygen uptake.

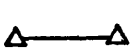
Is there actually an important difference in response determined by the initial P_{O_2}? (See Figure 8.2.2.). Consideration of values for the initial rate of oxygen uptake at high and low P_{O_2} does suggest a difference here. However, taking the values at 14μM oxygen after having started the experiment at 220μM does provide a better comparison with rates starting at 14μM oxygen. The principle difference here concerns again BSA when comparing conditions using the same tissue

FIGURE 8.2.2. COMPARISON OF INITIAL RATES OF SYNAPTOSOMAL OXYGEN UPTAKE FOR INCUBATIONS STARTING AT HIGH AND LOW OXYGEN CONCENTRATIONS

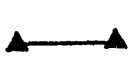
KEY

P_2B (synaptosomes)

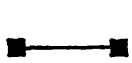
P_2B + glucose (10mM)



P_2B + BSA (3.33mg/ml)



P_2B + glucose + BSA (3.33mg/ml)



P_2B + BSA (13.33mg/ml)

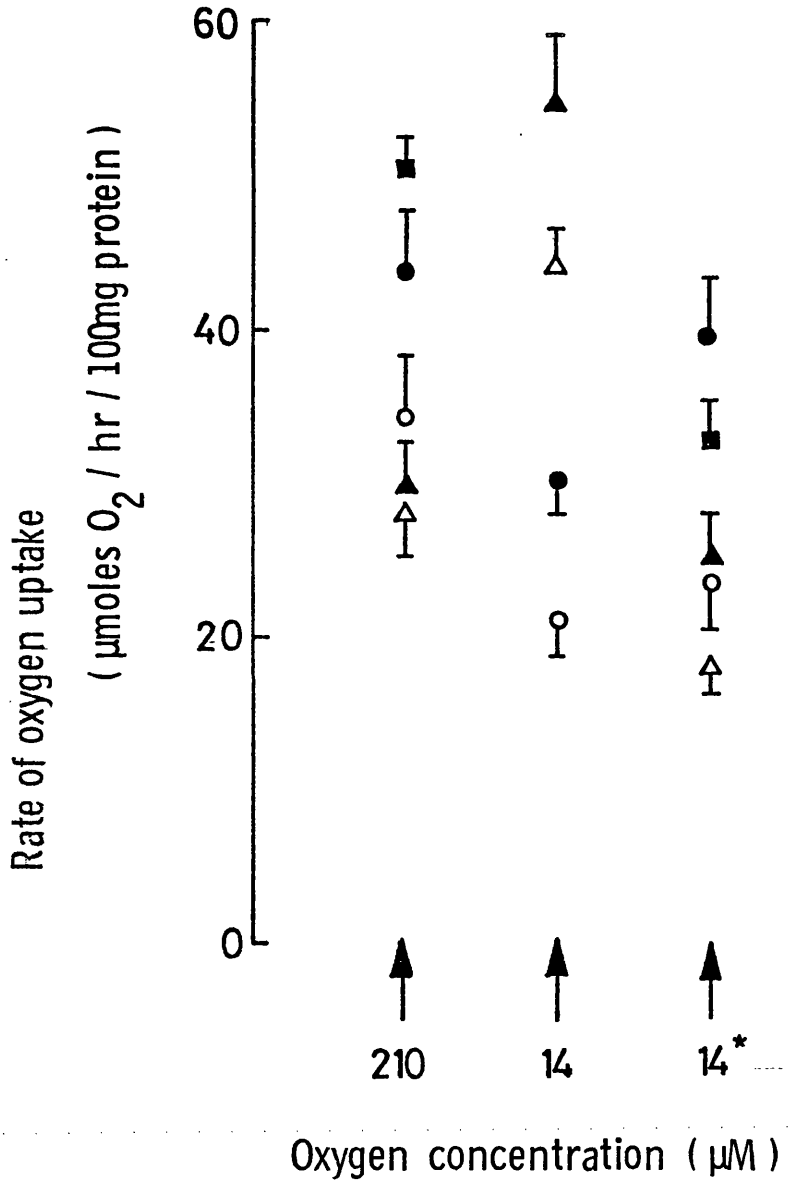
14^* Value at 14μM-O_2 after starting incubation at 220μM-O_2

Measured at 30°C in Krebs phosphate medium.

Data taken from Figs. 8.2.1.(i) and 8.2.1.(ii).

Values are Mean ± S.E.M.

FIGURE 8.2.2.


60
40
20
0
Rate of oxygen uptake
(µmoles O_2 / hr / 100mg protein)
210
14
14*
Oxygen concentration (µM)

to BSA ratio. The rate starting at 14μM oxygen was significantly higher ($P<0.01$) than expected and conversely, the rate with glucose appeared to be lower, although this difference was not statistically significant.

8.2.3. The effect of pre-incubating synaptosomes in a shaking water bath before measuring oxygen uptake at low oxygen tensions

Brain tissue in vitro is routinely pre-incubated before examining experimental conditions in order to restore, for example, the levels of K^+ and ATP (McIlwain & Bachelard, 1971). Marchbanks (1975) has suggested that synaptosomes should be pre-incubated for 15 minutes before using them in transport studies.

The following pre-incubation experiments have been performed in order to establish the effects of pre-incubation on respiration and particularly to investigate the cause of the different rates of oxygen uptake found at 14μM oxygen - after starting at 220μM - compared with experiments starting at 14μM oxygen. Also related here is the observation that the rate of synaptosomal oxygen uptake gradually diminishes with time after preparation and the long preparation time itself exposes the tissue to considerable periods of anoxia.

Synaptosomes placed in air-saturated Krebs phosphate medium take over 25 min to consume sufficient oxygen to reduce the concentration to 14μM. For the following experiments, the resuspended synaptosomal pellet was pre-incubated for 35 min at 30°C in an open vessel before measuring the rate of oxygen uptake at low P_{O_2}. Glucose and/or BSA were added to the pellet and/or the oxygen electrode chamber. The procedure is described in more detail in Section 8.1.3. and the results are given in Table 8.2.3. which shows the rate of oxygen uptake when the oxygen concentration had reached 7μM, after starting at an initial value of 14μM.

TABLE 8.2.3. The effect of pre-incubating synaptosomes in a shaking water bath before measuring oxygen uptake at low oxygen tensions

Condition	n.	Rate of O_2 uptake	Significance level compared to: Krebs control	Own control
No pre-incubation				
Krebs CONTROL	5	15.49(1.65)		
G(c)	5	26.64(2.70)	P<0.01	
B(c)	5	35.53(1.75)	P<0.001	
G + B (c)	5	47.09(4.00)	P<0.001	
Pre-incubation, shaking				
Krebs	5	7.28(0.90)	P<0.05	
G(c)	5	11.60(0.92)	P<0.05	P<0.05
G(p)	5	19.92(3.57)	NS	P<0.01
G(p + c)	9	16.20(1.34)	NS	P<0.001
B(c)	5	13.50(1.82)	NS	P<0.05
B(p)	5	10.04(0.96)	P<0.05	NS
B(p + c)	5	17.96(2.21)	NS	P<0.01
G + B(c)	5	20.84(3.10)	NS	P<0.01
G + B(p)	5	14.62(1.97)	NS	P<0.01
G + B(p + c)	5	15.55(1.79)	NS	P<0.01

All incubations were at 30°C with a pre-incubation time of 35min. Control tissue was kept on ice. Values were taken when the P_{O_2} had reached 7μM after starting at an initial P_{O_2} of 14μM. For explanation of the abbreviations see Table 8.1.3. Values for respiratory activity are expressed as μmoles O_2/hr/100mg protein; Mean (S.E.M.).

Firstly consider the results obtained with all the pre-incubations using Krebs phosphate medium and with the additions made to the oxygen electrode chamber. All the responses are present as in the control experiments, i.e. oxygen uptake is increased in the following order; glucose, BSA, and glucose plus BSA. However all the results are proportionately reduced.

The presence of glucose during the pre-incubation period does not bring the rate up to that at 14μM oxygen (from 220μM) and similarly the presence of BSA is not comparable with the previous BSA results (starting from 220μM).

8.2.4. The effect of pre-incubating synaptosomes in a non-shaking water bath before measuring oxygen uptake at low oxygen tensions

The method used is described above (8.2.3.) and the results are given in Table 8.2.4.

TABLE 8.2.4. The effect of pre-incubating synaptosomes in a non-shaking water bath before measuring oxygen uptake at low oxygen tensions

Condition	n.	Rate of O_2 uptake	Significance level compared to: Krebs control	Own control
No pre-incubation				
Krebs CONTROL	5	15.49(1.65)		
G(c)	5	26.64(2.70)	P<0.01	
B(c)	5	35.53(1.75)	P<0.001	
G + B(c)	5	47.09(4.00)	P<0.001	
Pre-incubation, non-shaking				
Krebs	5	13.88(1.87)	NS	
G(c)	5	15.70(0.99)	NS	NS
G(p)	11	15.42(1.09)	NS	NS
G(p + c)	10	14.25(1.70)	NS	NS
B(c)	4	5.80(0.88)	P<0.01	P<0.01
B(p)	6	5.53(0.63)	P<0.001	P<0.01
B(p + c)	6	7.13(0.62)	P<0.01	P<0.01
G + B(c)	4	11.16(1.54)	NS	NS
G + B(p)	6	10.99(1.35)	NS	NS
G + B(p + c)	4	28.65(10.99)	NS	NS

All incubations were at 30°C with a pre-incubation time of 35min. Control tissue was kept on ice. Values were taken when the P_{O_2} had reached 7μM after starting at an initial P_{O_2} of 14μM. For explanation of the abbreviations see Table 8.1.3. Values for respiratory activity are expressed as μmoles O_2/hr/100mg protein; Mean (S.E.M.).

In contrast to the results obtained using a shaking water bath, the addition of glucose to the oxygen electrode chamber did not stimulate oxygen uptake after pre-incubation in a non-shaking water bath. The addition of BSA now significantly reduces oxygen uptake although the presence of glucose with BSA does seem to counteract the reduction caused by BSA.

Of interest here in particular is the observation that the control rate after the pre-incubation is not significantly different from the control rate of synaptosomes kept on ice.

The presence of glucose or BSA during the pre-incubation again is not comparable with the results at 14μM oxygen after starting the measurements at 220μM.

8.2.5. Comparison of the effects of pre-incubating synaptosomes in either a shaking or a non-shaking water bath

When considering the influence of glucose or BSA after the pre-incubation process, a similar result appears despite the apparent contradictory responses. That is to say that the results in the presence of glucose plus BSA are strikingly similar to the combined effects of individual components whether or not, for example, BSA produces a stimulation or a diminution in the rate of oxygen uptake by the synaptosomes.

Pre-incubation of the synaptosomes in the presence of glucose or BSA has not provided an obvious answer to the results obtained starting at either high or low P_{O_2}. (Summarised in Figure 8.2.5.).

Two further points need to be considered before a more complete discussion of these results can be made.

(i) A few experiments were made by pre-incubating synaptosomes for 35 min at 30°C and subsequently measuring oxygen uptake in air-saturated medium. The results in Table 8.2.5.(i) were taken midway through the course of measuring oxygen uptake (110μM-O_2) and here we see that although both types of pre-incubation significantly reduced the rates ($P<0.001$), the results within each group were similar to those of Sections 8.2.3. and 4. at low P_{O_2}. With glucose present during pre-incubation in a shaking water bath, the rate of oxygen uptake was significantly increased ($P<0.01$) compared to that in its absence.

Table 8.2.5.(i). The effect of pre-incubating synaptosomes before measuring oxygen uptake at high oxygen tensions

Condition	n.	Rate of O_2 uptake	Significance level compared to: Krebs control	Own control
Krebs CONTROL	7	33.19(1.21)		
Pre-incubation, shaking				
Krebs	5	14.19(1.51)	P<0.001	NS
G(p)	5	22.15(1.52)	P<0.001	P<0.01
Pre-incubation, non-shaking				
Krebs	5	19.23(2.13)	P<0.001	NS
G(c)	5	23.72(1.65)	P<0.001	NS
G(p)	5	23.96(0.87)	P<0.001	NS

All incubations were at 30°C with a pre-incubation time of 35 min. Values were taken when the P_{O_2} had reached 110μM after starting at an initial P_{O_2} of 220μM. For explanation of the abbreviations see Table 8.1.3. Values for respiratory activity are expressed as μmoles O_2/hr/100mg protein; Mean (S.E.M.).

(ii) Despite changes in pestle sizes, the results obtained have been reproducible. However when the results obtained using Method 6 are considered (which was the short teflon pestle but whose preparation of synaptosomes varies slightly (see Section 4.5.), a different picture emerges.

The results expressed in Table 8.2.5.(ii) show that in the control experiments at low P_{O_2}, the glucose stimulation response is absent and the BSA effect is not so marked. In the pre-incubation experiments also there are no significant differences from the control experiments and no differences within each group. A possible explanation for this discrepancy is discussed in Section 11.

TABLE 8.2.5.(ii). The influence of a different preparation (Method 6) on the rate of oxygen uptake by synaptosomes at low oxygen tensions

Condition	n.	Rate of O_2 uptake
Krebs CONTROL	17	21.79(1.79)
G(c)	23	21.89(1.37)
B(c)	12	26.34(2.37)
Pre-incubation, shaking		
Krebs	5	18.86(3.50)
G(c)	5	21.78(3.35)
B(c)	5	22.68(2.68)
Pre-incubation, non-shaking		
Krebs	5	18.80(1.64)
G(c)	5	15.84(2.37)
B(c)	5	17.64(2.95)

All incubations were at 30°C with a pre-incubation time of 35 min. Values were taken when the P_{O_2} had reached 7μM after starting at an initial P_{O_2} of 14μM. For explanation of the abbreviations see Table 8.1.3. Values for respiratory activity are expressed as μmoles O_2/hr/100mg protein; Mean (S.E.M.).

FIGURE 8.2.5. COMPARISON OF SYNAPTOSOMAL OXYGEN UPTAKE AT LOW OXYGEN CONCENTRATIONS BEFORE AND AFTER PRE-INCUBATION

KEY

o o P_2B (synaptosomes)

● ● P_2B + glucose (10mM)

△ △ P_2B + BSA (3.33mg/ml)

_____ Control conditions (tissue stored on ice)

- - - - - Non-shaking pre-incubation (35 min at 30°C)

_ . _ . _ . _ Shaking pre-incubation (35 min at 30°C)

Measured at 30°C in Krebs phosphate medium. The points at 28μM oxygen represent the rate of synaptosomal O_2 uptake after starting the measurements in air-saturated medium. All other measurements taken after placing the synaptosomes in a medium of low O_2 concentration (14μM).

For the pre-incubation experiments, glucose or BSA were present during the pre-incubation, and for the control experiments they were present in the oxygen electrode chamber. See Section 8 for full details.

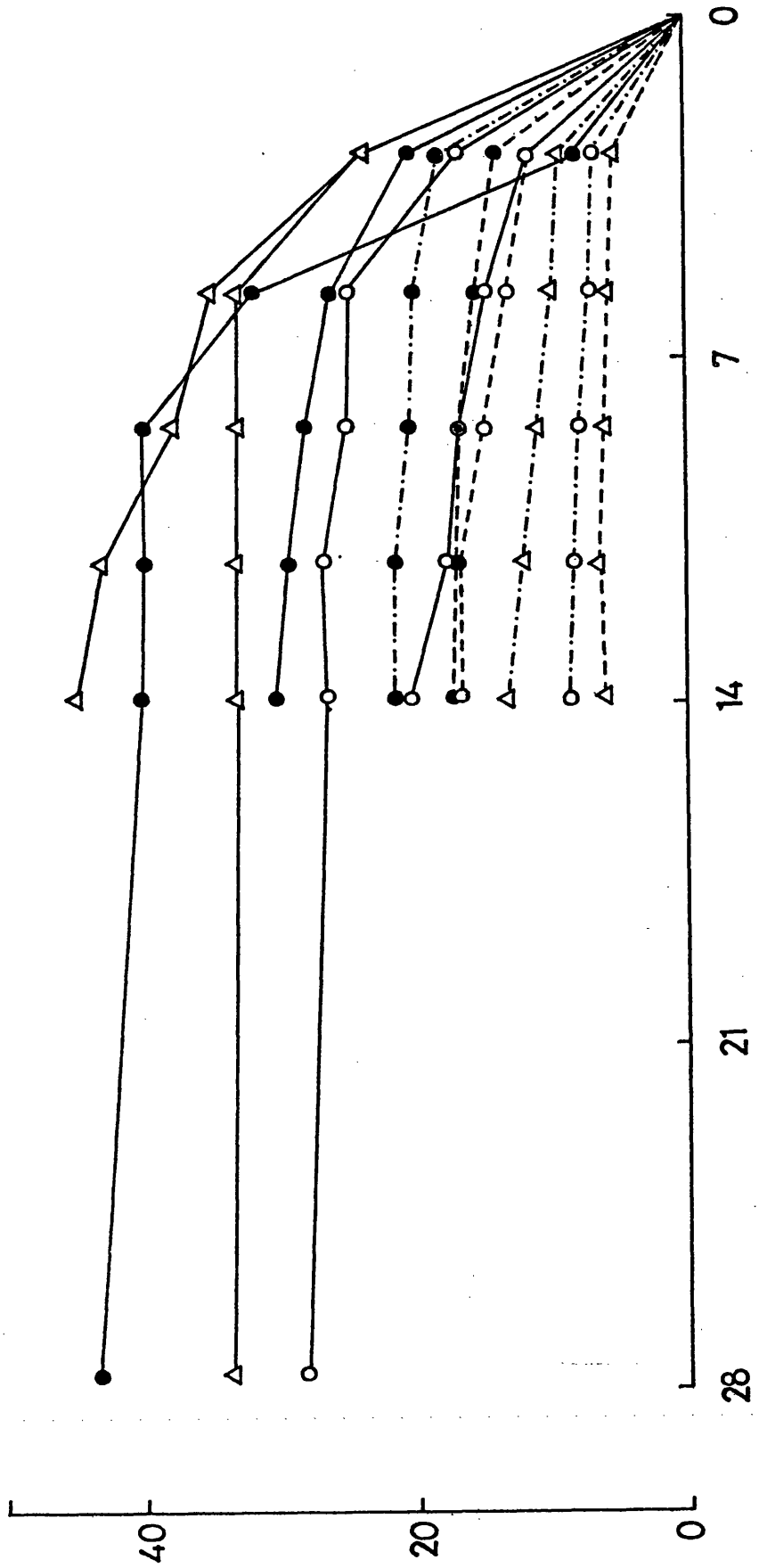

Rate of oxygen uptake
(μmoles O_2 / hr / 100mg protein)
40
20
0
0
7
14
21
28
Oxygen concentration (μM)

FIGURE 8.2.5.

8.2.6. The effect of pre-incubation on the lactate dehydrogenase activity of synaptosomes

The different results obtained using a shaking or a non-shaking water bath may be a reflection of varying degrees of disruption of the synaptosomes by (a) an increase in temperature, and (b) mechanical disruption (Marchbanks, 1967). Disruption of the synaptosomal membrane can be estimated by measuring the proportion of free to occluded lactate dehydrogenase (LDH). An increase in the proportion of free LDH activity reflects a loss of synaptosomal membrane integrity.

Table 8.2.6.(i) gives the specific activity of LDH in synaptosomes pre-incubated with and without 10mM glucose. LDH was assayed according to Kornberg (1955) as described in Section 4.4.

Table 8.2.6.(ii) expresses these results as a percentage of total activity in order to indicate more clearly whether or not there are any differences between the two forms of pre-incubation.

TABLE 8.2.6.(i). The effect of pre-incubation on the lactate dehydrogenase activity of synaptosomes

Condition	n.	Specific Activity of LDH	
CONTROL			
Krebs	8	0.568(0.054)	Total
		0.123(0.022)	Free
		0.444(0.059)	Occluded
Pre-incubation, shaking			
Krebs	7	0.529(0.133)	Total
		0.162(0.052)	Free
		0.367(0.097)	Occluded
10mM glucose	5	0.534(0.055)	Total
		0.206(0.033)	Free
		0.327(0.064)	Occluded
Pre-incubation, non-shaking			
Krebs	5	0.529(0.135)	Total
		0.169(0.050)	Free
		0.360(0.094)	Occluded
10mM glucose	5	0.553(0.075)	Total
		0.211(0.027)	Free
		0.342(0.057)	Occluded

Control samples kept on ice. Pre-incubation for 35 min at 30°C with the synaptosome pellet resuspended in Krebs phosphate medium containing 10mM glucose where indicated. Results are expressed as Mean (S.D.).

TABLE 8.2.6.(ii). The effect of pre-incubation on the proportion of free lactate dehydrogenase activity in synaptosomes

Condition	n.	LDH activity as % TOTAL		Significance level compared to:
		FREE	OCCLUDED	CONTROL
CONTROL	8	21.89(4.36)	77.99(4.32)	
Pre-incubation				
Shaking control	7	30.63(6.23)	69.37(6.23)	$P<0.01$
Non-shaking control	5	31.97(4.90)	68.03(4.90)	$P<0.05$
Shaking + glucose	5	39.03(7.57)	60.97(7.57)	$P<0.001$
Non-shaking + glucose	5	38.36(3.71)	61.64(3.71)	$P<0.001$

Where stated, 10mM glucose was present during the pre-incubation period of 35 min at 30°C. Results are expressed as Mean (S.D.).

Although pre-incubation in the absence of added glucose or BSA, under shaking and non-shaking conditions, significantly increased the proportion of free to occluded LDH when compared to tissue kept on ice, there was no difference between the two types of pre-incubation (see Table 8.2.6.(ii)). The presence of glucose during the pre-incubation resulted in a further increase in the proportion of free LDH activity.

9. FURTHER STUDIES ON THE INFLUENCE OF BSA AND CHANGES IN THE IONIC ENVIRONMENT ON OXYGEN UPTAKE BY SYNAPTOSOMES PLACED IN MEDIA OF LOW OXYGEN TENSION.

9.1. METHODS

9.1.1. Preparation of synaptosomes

Rat cerebral cortex synaptosomes were prepared by Method 6 as described in Section 3, based on Bradford et al. (1975).

9.1.2. Media

Krebs phosphate medium (Bradford et al., 1975). Composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 0.75; $MgSO_4$, 1.3; Na_2HPO_4, 20 at pH 7.8.

Where stated as Ca^{2+}-free, this refers to the exclusion of $CaCl_2$ from the Krebs phosphate medium. Various additions of EGTA were also made to chelate Ca^{2+}, EGTA forms a more stable chelate with Ca^{2+} and a much weaker chelate with Mg^{2+} than does EDTA (West & Sykes, 1960).

9.1.3. Experimental design

In the following low P_{O_2} experiments, 100µl samples of synaptosomes (1.27mg protein, S.E.M. = 0.23, n = 211) were added to 2.9ml of medium of reduced oxygen concentration to give a final oxygen concentration of 14µM in the oxygen electrode chamber. All tissue was kept on ice and measurements of oxygen uptake were made at 30°C. If a Ca^{2+}-free medium was to be used in the oxygen electrode chamber, then Ca^{2+}-free medium was also used to resuspend the synaptosomal pellet.

9.2. RESULTS

9.2.1. The influence of BSA on synaptosomal oxygen uptake at low oxygen tensions

The use of BSA throughout the experiments described so far has been continued due to its possible effects on mitochondrial function.

Two seemingly contradictory reports suggest that (1) the presence of BSA removes free fatty acids, particularly unsaturated long chain fatty acids which have been shown to be potent uncouplers of oxidative phosphorylation (Chan & Higgins, 1978). (2) Free fatty acids have been shown to lower oxygen uptake in mitochondria, (Lochner et al., 1976) possibly by causing the leakage of essential cofactors such as NAD^+, cytochrome c, and CoA.

To determine whether the removal of free fatty acids (FFA) from commercial BSA had any effect on synaptosomal oxygen uptake, a preparation of fatty acid-free BSA was obtained from Sigma Chemical Co. Ltd.

BSA has a strong affinity for anions and has been found to markedly affect cardiac function, an effect suspected of being due to the binding of Ca^{2+} (M.J. Parry, personal communication). For this reason, Ca^{2+} was excluded from the Krebs phosphate medium and various additions of EGTA were used, see Table 9.2.1.

TABLE 9.2.1. The influence of BSA on synaptosomal oxygen uptake at low oxygen tensions

Additions	n.	Rate of O_2 uptake	Significance level compared to CONTROL
Krebs CONTROL	6	22.69(1.08)	
BSA (3.33mg/ml)	5	32.96(2.20)	P<0.01
Fatty acid free BSA	6	34.49(2.77)	P<0.01
1mM EGTA	6	27.09(1.67)	NS
Ca^{2+}-free + 0.5mM EGTA	4	24.38(2.64)	NS
Ca^{2+}-free + 1mM EGTA	5	36.81(3.42)	P<0.01

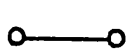
Synaptosomes at 30°C in Krebs phosphate medium. Values were taken when the P_{O_2} had reached 7μM after starting at an initial P_{O_2} of 14μM. Values for respiratory activity are expressed as μmoles O_2/hr/100mg protein; Mean (S.E.M.).

Apart from observing that both BSA and fatty acid-free BSA have identical effects on the rate of oxygen uptake by synaptosomes, these results show the sensitivity of this process to the presence of Ca^{2+}. The use of a Ca^{2+}-free medium plus EGTA to complex membrane bound calcium produces a significant increase in the rate of oxygen uptake equal in magnitude to the presence of BSA. Bradford et al. (1973) also found a substantial increase in rates of respiration when calcium was omitted from the medium.

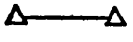
Whether or not the observed responses of synaptosomes to BSA are due to the binding of Ca^{2+} remains to be determined, but the results from my experiments indicate that this may be so.

FIGURE 9.2.1. THE INFLUENCE OF BSA AND A Ca^{2+}-FREE MEDIUM ON SYNAPTOSOMAL OXYGEN UPTAKE AT LOW OXYGEN CONCENTRATIONS

KEY

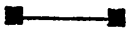


P_2B (synaptosomes)



P_2B + BSA (3.33mg/ml)

P_2B + fatty acid-free BSA (3.33mg/ml)

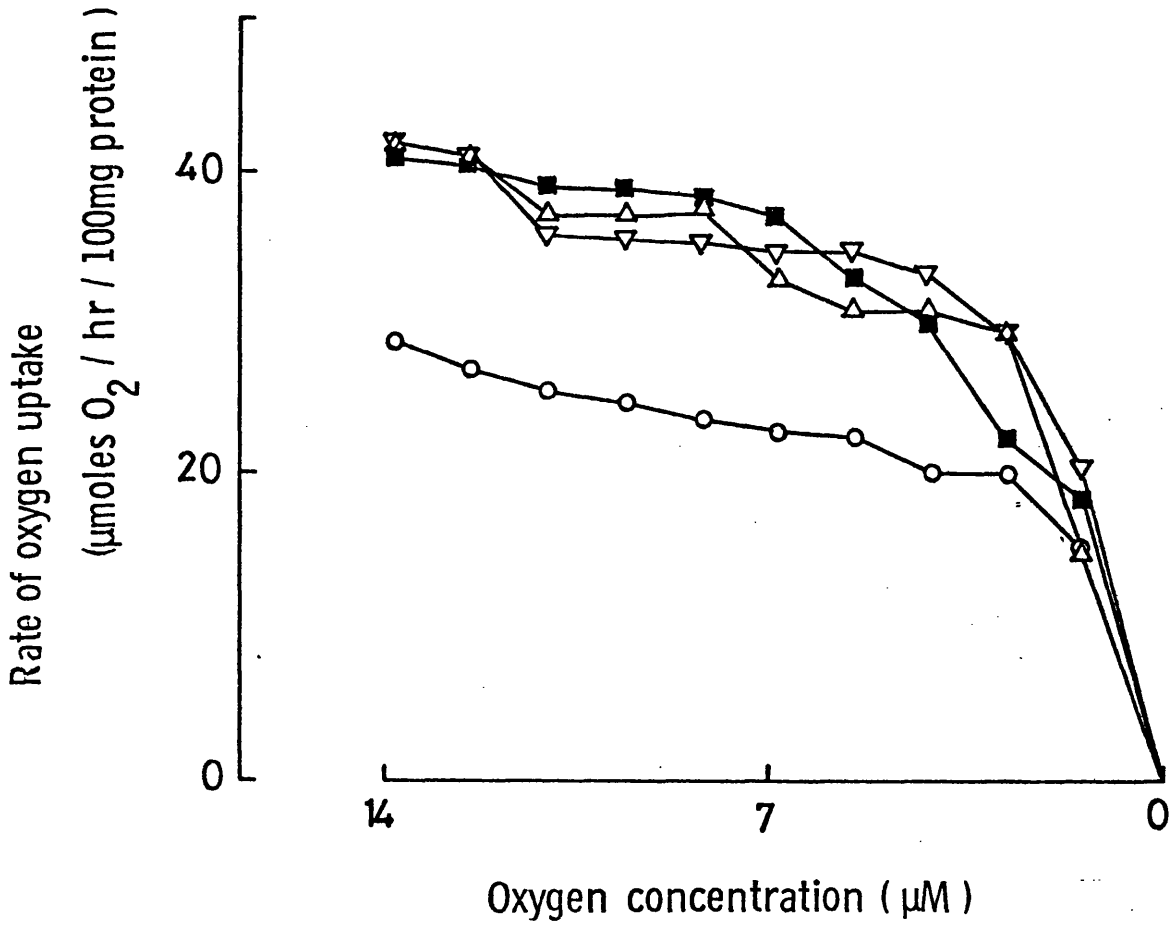


P_2B + Ca^{2+}-free medium containing 1mM-EGTA

Measured at 30°C in Krebs phosphate medium. (n = 5-6).

The ratio of tissue (mg) to BSA (mg) was 1.27 : 10.

FIGURE 9.2.I.


Rate of oxygen uptake
(μmoles O_2 / hr / 100mg protein)
40
20
0
14
7
0
Oxygen concentration (μM)

9.2.2. The influence of the ionic environment on oxygen uptake by synaptosomes at low oxygen tensions

As already indicated in the preceding section and in Section 6, the ionic environment of the synaptosomes strongly influences their behaviour. An accidental finding showed that reduction of the Na_2HPO_4 concentration in the Krebs phosphate medium to a concentration 39% of normal, whilst not affecting control rates of oxygen uptake, did prevent the stimulation normally seen on the addition of glucose or BSA.

Although the sample numbers are low here, a consistent trend is found after pre-incubating the tissue at 30°C in a non-shaking water bath for various time intervals, see Table 9.2.2.

TABLE 9.2.2. The influence of a reduced concentration of Na_2HPO_4 on oxygen uptake by synaptosomes at low oxygen tensions

Condition	n.	Rate of O_2 uptake
Krebs	10	23.98(1.27)
10mM glucose	3	22.90(1.45)
BSA (3.33mg/ml)	3	16.68(3.49)
Pre-incubation, non-shaking		
5 min.	3	14.16(1.71)
10 min.	2	12.08(2.49)
35 min.	3	8.05(0.70)
35 min. + 10mM glucose in O_2 electrode chamber	3	12.43(2.98)

Measured at 30°C in Krebs phosphate medium of reduced PO_4^{3-} content. Values were taken when the P_{O_2} had reached 7μM after starting at an initial P_{O_2} of 14μM. Values for respiratory activity are expressed as μmoles O_2/hr/100mg protein; Mean (S.E.M.).

A pre-incubation time of only 5 min was sufficient to produce a highly significant($P<0.001$) fall in the rate of oxygen uptake by synaptosomes. A further significant reduction ($P<0.05$) is seen on extending the pre-incubation time from 5 to 35 min. This decrease in the rate of oxygen uptake by approximately 66% has not been found in any of the experiments involving non-shaking pre-incubation in a normal Krebs phosphate medium.

10. THE USE OF AN OXYSTAT TO STUDY THE METABOLISM OF SYNAPTOSOMES

10.1. METHODS

10.1.1. Preparation of synaptosomes

Rat cerebral cortex synaptosomes were prepared by Method 6 as described in Section 3, based on Bradford et al. (1975).

10.1.2. Media

Krebs phosphate medium (Bradford et al., 1975). Composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 0.75; $MgSO_4$, 1.3; Na_2HPO_4, 20 at pH 7.8.

10.1.3. Experimental design

The Rank oxygen electrode was adapted for use as an oxystat as described in Section 5.2. Three oxygen tensions were used to reflect normoxia, hypoxia and anoxia.

For conditions of normoxia, 2.9ml of Krebs phosphate medium at 30°C was added to the oxygen electrode chamber, the gas outlet was lowered into position and the chamber sealed with Parafilm. With the stirrer on, the P_{O_2} of the medium was monitored on the chart recorder and pure air was blown over the surface of the stirred medium. When a constant P_{O_2} was reached, 100µl(∿ 1.27mg protein) of synaptosomes were added to the medium using a Hamilton syringe. The final oxygen concentration will be 200-220µM depending on the respiratory activity of the tissue.

For conditions of hypoxia, the final oxygen concentration is required to be approximately 14µM. By mixing N_2 with air an oxygen concentration of close to 7µM was set before the addition of the synaptosomes which would initially increase the P_{O_2} due to the oxygen dissolved within the suspension. The mixture of N_2 and air was then controlled manually in order to maintain the concentration of oxygen

at or below 14μM.

For conditions of anoxia, N_2 only was blown over the stirred medium until the pen registered zero oxygen. The introduction of the synaptosomes would add a small amount of dissolved oxygen but this will be rapidly depleted and the pen remain at zero.

In all cases the zero time point was taken as the time of introduction of the synaptosomes to the medium. In the experiments described below, synaptosomes were incubated as defined in each section and the incubations were terminated either by removing the suspensions for the immediate assay of LDH activity or by removal of a sample with immediate fixation using perchloric acid for the subsequent assay of lactate. For the extraction of free fatty acids (FFA), the tissue and medium were rapidly separated by filtration at the end of the incubation, (see Section 10.1.6.).

10.1.4. Fixation of tissue

The following sequence was used to fix tissue at a defined time point by precipitating proteins and lipids and thus preventing further metabolism (McIlwain, 1975).

To 3ml synaptosomal suspension add 0.1ml 71% (w/v) ice-cold, perchloric acid.

Stand on ice for 45 min.

Spin in bench centrifuge for 5 min.

Remove supernatant and keep at 0-4°C.

Resuspend pellet in 0.5ml ice-cold H_2O, recentrifuge.

Pool supernatants.

Add 2M KOH to bring pH to 6-7.

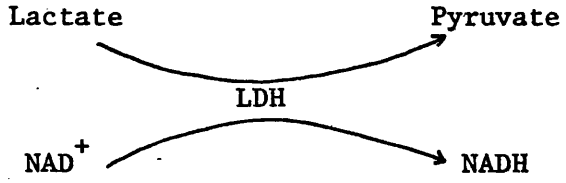
Stand for at least 10 min.

Remove precipitate by centrifugation.

Freeze neutralized extract.

10.1.5. Lactate assay

Lactic acid is assayed according to Berg meyer (1965), by monitoring the production of NADH by a sample containing added lactate dehydrogenase and an excess of NAD^+.



The amount of NADH produced is equivalent to the amount of lactate in the sample and consequently the amount of lactate originally present can be calculated from the absorption changes produced by standard solutions of lactate.

10.1.6. Filtration system

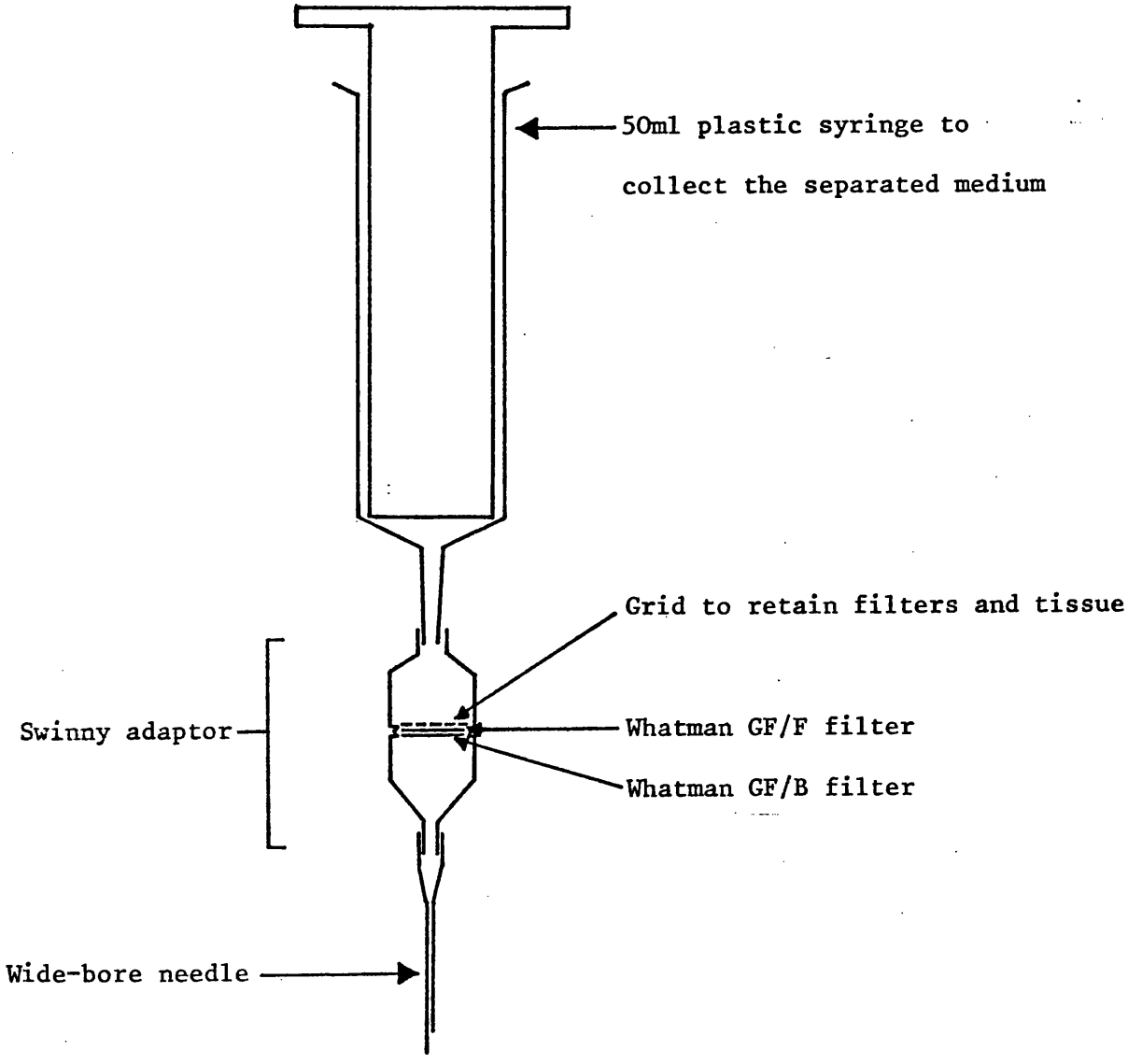
A method was devised to separate rapidly the synaptosomes from the incubation medium at the end of an experimental run in the oxygen electrode chamber.

It was necessary to make this separation directly from the oxygen electrode chamber so that there was little time for the suspension at reduced oxygen tensions to equilibrate to atmospheric P_{O_2}.

Filtration was achieved using Whatman glass fibre filters held in a Swinny adaptor interposed between a syringe needle and a 50ml plastic syringe. Initial experiments used a GF/B type filter (soaked in medium) and indicated that $\sim$ 92% of the tissue was retained by the filter. Further experiments showed that after an incubation time of 8 min, only 76% of the tissue was retained. A combination of two filters was found to be most efficient for separating incubated tissue - GF/B and GF/F with the latter adjacent to the syringe barrel, see Figure 10.1.6. To remove the total contents of the oxygen

FIGURE 10.1.6. FILTRATION APPARATUS FOR THE RAPID SEPARATION OF A SYNAPTOSOMAL SUSPENSION

(Not to scale)



electrode chamber, it was found necessary to withdraw the syringe plunger to its full extent and hold it there for 30 sec in order to maintain the suction and produce the desired separation.

This combination of two glass fibre filters still allows 8% of the protein to appear in the filtrate. The efficiency of the system could be increased by the use of an even finer filter since the GF/F filter has a 98% retention efficiency for particles only down to 0.7μM in liquids (Whatman, Ltd., 1979). The GF/B filter serves as a "pre-filter" retaining particles of 1.0μM size - mean synaptosome diameter is 0.5μM (Bradford, 1972).

10.1.7. Extraction and measurement of free fatty acids

Tissue samples, i.e. synaptosomes on glass-fibre filters, were extracted with chloroform-methanol (2:1, v/v) according to Lunt & Rowe (1968) and the samples of separated medium, were acidified with concentrated HCl before extraction with chloroform (Dole & Meinertz, 1960). The concentrated extracts were suspended in 1ml diethyl ether for methylation using diazomethane.

Methylation

Although distillation of diazomethane is hazardous (Fales et al. 1973), preliminary experiments indicated that undistilled diazomethane was unsuitable for the methylation of small samples of free fatty acids due to the introduction of contaminants as suggested by Schlenk & Gellerman (1960).

Double-distilled diazomethane in ether was therefore used and was freshly prepared from the addition of 0.5g KOH dissolved in 4.5 ml 90% ethanol to 2.4g N-methyl-p-toluenesulphonylnitrosamide in 50ml diethyl ether.

Approximately 10 drops of this mixture were added to each sample for 20 min before its removal by evaporation. The fatty acid methyl esters were stored at -30°C in petroleum ether, 40°-60°, before measurement by gas-liquid chromatography (GLC).

Gas-liquid chromatography

The methyl esters of the free fatty acids were analysed using a Tracor 550 gas chromatograph with the following specifications:

Column : 10% poly (diethyleneglycol succinate)

Column temperature : 200°C

Carrier gas flow : approx. 40ml/min

Detector : flame ionization

Methyl heptadecanoate was used as an internal standard. The areas of the peaks were measured by triangulation and calculations were made on the assumption that the areas were proportional to the mass of ester. Peaks were identified using fatty acid standards.

An estimation of recovery was made using a stock solution of palmitic acid dissolved in n-propyl alcohol and diluted with Krebs phosphate medium. Recoveries varied widely (Mean = 41%) and therefore corrections have not been made. It would perhaps be more useful to include an internal standard with the samples under the conditions used here, although there is no reason to assume that the recovery of the other acids will be identical.

10.2. RESULTS

10.2.1. Do synaptosomes become damaged during incubation in the oxystat?

To determine whether or not synaptosomes would become damaged during incubation in the oxystat, 400µl samples of synaptosomes (∿ 5mg protein) were incubated with 2.6ml Krebs phosphate medium for 30 min at 30°C and at an oxygen concentration of 140µM. Control samples were kept on ice and some were resuspended in Krebs phosphate medium containing 100mM sucrose to determine if this helped to stabilize the synaptosomal membranes and prevent leakage of LDH (Sperk & Baldessarini, 1977). LDH activity was measured as described in Section 4.4.

TABLE 10.2.1. The activity of lactate dehydrogenase in synaptosomes incubated in the oxystat

Condition	n.	Specific activity of LDH	% TOTAL	
Krebs, control	8	0.568(0.054)		Total
		0.123(0.022)	21.7	Free
		0.444(0.059)	78.2	Occluded
Krebs + 100mM sucrose,		0.593(0.073)		Total
control	5	0.142(0.030)	24.0	Free
		0.451(0.061)	76.0	Occluded
Incubation at 140μM O_2	5	0.890(0.120)		Total
		0.211(0.026)	23.7	Free
		0.679(0.120)	76.3	Occluded
Incubation at 140μM O_2		0.774(0.094)		Total
+ 100mM sucrose	5	0.210(0.055)	27.1	Free
		0.585(0.059)	75.5	Occluded

Control tissue was kept on ice, and incubated tissue at 140μM O_2, for 30 min at 30°C. 100mM sucrose was added to the medium where indicated. Values are Mean (S.D.).

Synaptosomes do not appear to be disrupted by incubation in the oxystat at high P_{O_2}, as no increase in the proportion of free LDH was found. Similarly the addition of 100mM sucrose was without effect suggesting that the membranes may already be stabilized by the sucrose already present from the density gradient step in the preparation. The total specific activity of LDH after incubation increased significantly in both Krebs phosphate medium and in the presence of 100mM sucrose ($P<0.001$ and $P<0.01$ respectively).

10.2.2. The influence of normoxia, hypoxia and anoxia on lactate production and the pH of a suspension of synaptosomes

100µl samples of synaptosomes (∿ 1.27mg protein) were incubated with 2.9ml Krebs phosphate medium in conditions of normoxia, hypoxia and anoxia as described in Section 10.1.3. At the end of a 10 min incubation period in the oxystat, a 1ml sample was removed and added to a tube containing ice-cold perchloric acid. Further processing of the tissue and assay of lactate has been described in Sections 10.1.4. and 5. A Radiometer micro-pH electrode was used to determine the pH of the remaining sample.

A Ca^{2+}-free medium was also used in which $CaCl_2$ had been omitted from the normal medium and 1mM EGTA added to complex membrane-bound calcium.

TABLE 10.2.2.(i). The influence of normoxia, hypoxia and anoxia on the pH of a suspension of synaptosomes

Condition	n.	Final pH	
		No glucose	10mM glucose
Normoxia	4,6	7.766(0.009)	7.733(0.014)
Hypoxia	6,6	7.768(0.012)	7.732(0.030)
Anoxia	6,6	7.767(0.016)	7.743(0.014)

Synaptosomes incubated in Krebs phosphate medium pH 7.8 for 10 min at 30°C. Values given are the final pH after the incubation period; Mean (S.D.).

TABLE 10.2.2.(ii). The influence of normoxia, hypoxia and anoxia on lactate production by a suspension of synaptosomes

Condition	n.	Total lactate	
		No glucose	10mM glucose
Normoxia	6	0.211(0.06)	0.179(0.07)
Hypoxia	6	0.150(0.08)	0.204(0.09)
Anoxia	6	0.194(0.09)	0.178(0.07)
Hypoxia, Ca^{2+}-free + 1mM EGTA	6	-	0.275(0.02)

Synaptosomes incubated in Krebs phosphate medium for 10 min at 30°C. Values for total lactate at the end of the incubation are expressed as μmoles lactate/mg protein; Mean (S.D.).

The results given in Tables 10.2.2.(i) and (ii) should compliment each other since an increased production of lactate is principally responsible for the fall in pH of a metabolising tissue. In terms of lactate production, the results are inconclusive due to large errors incurred here. However the removal of Ca^{2+} significantly increased lactate production compared to normoxia minus glucose ($P<0.05$), normoxia plus glucose ($P<0.01$) and hypoxia minus glucose ($P<0.01$). This reflects the increased respiratory activity seen in the oxygen uptake experiments in Section 9.2.1.

Fortunately the measurement of pH changes proved more successful and show that at all oxygen tensions, the presence of 10mM glucose significantly decreases the final pH value after the 10 min incubation period. The degrees of significance comparing the addition of glucose

are: Normoxia, $P<0.001$; Hypoxia, $P<0.05$; Anoxia, $P<0.05$. There are no significant differences between the three oxygen tensions used.

10.2.3. The influence of normoxia, hypoxia and anoxia on the release of free fatty acids by a suspension of synaptosomes

Experiments were conducted in the same manner as described in Section 10.1.3. using 100µl samples of synaptosomes (∿ 1.27mg protein) with 2.9ml Krebs phosphate medium. At the end of a 10 min incubation at 30°C in the oxystat, the tissue and medium were rapidly filtered within 30 sec by glass filters as described in Section 10.1.6.

Free fatty acids (FFA) were extracted from the tissue and medium samples according to the method described in Section 10.1.7. and subsequently measured by GLC.

Glucose was present throughout the 10 min incubation period for all the conditions studied here, see Table 10.2.3.(a).

Table 10.2.3.(a). The free fatty acid composition of a synaptosomal suspension after incubation with glucose in conditions of normoxia, hypoxia and anoxia.

	Synaptosomes	Medium			Synaptosomes	Medium
C16:0	2.98(0.71)	3.16(0.73)	N	C18:0	4.00(1.09)	2.04(0.39)
	2.19(0.73)	2.01(0.19)	H		2.14(0.45)	1.53(0.45)
	2.92(0.17)	5.27(2.63)	A		2.52(0.58)	2.67(0.37)
C18:1	6.90(0.91)	8.01(2.33)	N	C18:2	5.08(0.57)	5.59(1.37)
	4.12(0.97)	2.83(0.48)	H		3.41(1.42)	2.99(0.79)
	7.00(0.84)	5.70(2.01)	A		5.13(0.57)	5.04(1.54)
C20:4	5.74(0.97)	6.45(0.89)	N	C_{OTHERS}	6.82(0.83)	6.87(0.51)
	3.55(0.77)	6.70(2.87)	H		4.24(0.66)	4.59(1.37)
	4.88(1.30)	6.43(1.37)	A		5.35(0.79)	5.62(1.28)

Synaptosomes were incubated in Krebs phosphate medium plus 10mM glucose for 10 min at 30°C. After incubation in normoxia (N), hypoxia (H) and anoxia (A), the suspension was rapidly filtered to separate the synaptosomes from the medium. Values given are µg free fatty acid/ mg protein, Mean (S.E.M.), n = 4. Methylating agent: distilled diazomethane.

The fatty acid species obtained were principally palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and arachidonic acid (C20:4). C_{OTHERS} represents the sum of three unidentified peaks positioned between C18:2 and C20:4. Lauric (C12:0) and myristic acid (C14:0) were also detected but those ran too close to the solvent peak for accurate determinations to be made.

The results expressed in Table 10.2.3.(a) indicate that neither incubation of synaptosomes at reduced or zero O_2 concentrations has any statistically-significant effect on the production and release of free fatty acids. This result is somewhat surprising since Bazán & Cummings (1969) reported a marked accumulation of FFAs after decapitation (ischaemia), and suggests that these experiments should be repeated in order to reduce the variation in results and that incubation times greater than 10 min might be more suitable. The total yield of free fatty acids from both tissue and medium was approximately 54µg/mg protein and it would be advantageous to use larger synaptosomal samples in future although the filtration system employed here would require some modifications.

When the data given in Table 10.2.3.(a) is expressed in terms of the relative distribution of each fatty acid with respect to the total recovered, i.e. synaptosomes plus medium, one can see that there is no preferential release of any fatty acid species into the medium. The distribution of free fatty acids is similar in both tissue and medium, and is constant in normoxia, hypoxia and anoxia when glucose is present. The unsaturated long-chain free fatty acids make the largest contribution to the total free fatty acids recovered, see Table 10.2.3.(b).

Table 10.2.3.(b). The distribution of free fatty acids from synaptosomal suspensions incubated with glucose in conditions of normoxia, hypoxia and anoxia

		Free fatty acids as % total recovered		
		Normoxia	Hypoxia	Anoxia
C16:0	T	4.63(0.67)	5.06(0.61)	5.00(0.30)
	M	4.95(1.02)	5.32(0.55)	9.16(4.75)
C18:0	T	5.97(1.23)	5.46(0.70)	4.35(1.06)
	M	3.33(0.62)	4.13(1.54)	4.52(0.53)
C18:1	T	10.91(0.72)	7.48(2.15)	12.06(1.64)
	M	12.01(2.50)	7.71(1.88)	9.53(3.19)
C18:2	T	8.11(0.53)	7.81(1.63)	8.80(1.07)
	M	8.53(1.38)	7.48(1.48)	8.48(2.41)
C_{OTHERS}	T	9.42(1.73)	9.22(2.31)	8.52(2.44)
	M	10.23(0.64)	11.12(4.53)	10.86(2.08)
C20:4	T	10.78(0.29)	11.58(2.14)	9.26(1.57)
	M	11.13(0.89)	10.76(2.82)	9.48(2.01)
Total	T	48.39(3.49)	49.32(4.58)	47.98(7.07)
	M	50.18(3.40)	50.69(4.58)	52.02(7.07)
Yield µg/mg protein		63.62(8.29)	40.30(8.53)	58.51(1.51)

Synaptosomes were incubated in Krebs phosphate medium plus 10mM glucose for 10 min at 30^{0}C, after which the suspension was rapidly filtered to separate the tissue (T) from the medium (M). Values given are Mean (S.E.M.), n = 4. Methylating agent: distilled diazomethane.

11. COMMENTS ON THE EXPERIMENTAL DESIGN

Several points have been highlighted in the preceding sections with reference to the accuracy and reproducibility of results - particularly concerning the measurement of syanptosomal oxygen uptake. These points are considered below:

1) Standardization of preparative procedures - the importance of this is brought out both in the characterization studies (see Section 4.5.) and also in the oxygen uptake studies. With reference to the latter, the results given in Section 7.3. clearly show the range of rates which can be produced simply by altering the initial homogenization procedure. Not only must the specifications of the pestle and tube be rigorously controlled but also the speed of homogenization and the number of up-and-down strokes of the rotating pestle. Also when diluting synaptosomes from the density gradient, standardized procedures must be used to avoid changes in tonicity influencing the final results (see Section 6.2.6.).

2) Since the control rate of oxygen uptake decreases slowly during the course of an experiment, one must rotate the different conditions under test to avoid the same conditions being studied at the beginning of an experiment and thereby producing relatively high results. For this reason also, synaptosomal tissue must be used up as rapidly as possible, ideally not more than 2 hr after obtaining the synaptosome pellet.

3) As found in Sections 6 and 9, the ionic environment strongly influences synaptosomal behaviour - with respect to the process of oxygen uptake - and it is felt that this is the likely cause for the discrepancies noted in Section 8.2.5. Does the Krebs phosphate medium used provide the optimal support for synaptosomal oxygen uptake?

Bradford & Ward (1975) have suggested the possible inclusion of glutamine to incubation fluids.

4) Should the tissue be incubated before experimental conditions are imposed and if so how? The results of Sections 8.2.3. and 4. clearly indicate that a synaptosomal suspension should be shaken whilst being incubated. Which of all the conditions studied here actually gives the closest representation of conditions in vivo? Here I would choose shaking pre-incubation in the presence of 10mM glucose.

5) Possible solutions to some of these problems are (a) pairing samples, e.g. 2 x 100µl samples can be taken from each resuspended synaptosomal pellet of which one would be used as the control and the other as the test sample. When both samples have been treated identically the deviation from the mean of the paired samples was calculated as 3.80µmoles O_2/hr/100mg protein (S.E.M., 0.85, n = 9). This difference would become more significant with less active preparations suggesting that a pestle of 3cm length and 0.25mm clearance should be the type of choice.

(b) Use larger sample volumes to reduce the influence of the electrode error (see Section 5.1.). This may however cause difficulties if the suspension is required to be rapidly filtered.

6) The overall impression gained suggests that the oxygen electrode is suited best for measuring rapid reactions with successive additions of trial substances (as in Section 6) and in particular for experiments where the P_{O_2} must be fixed as in the oxystat (Section 10). Longer experiments in oxygenated media may be best studied using Warburg flasks as in Bradford (1969).

CHAPTER 3

DISCUSSION

Chapter 3. DISCUSSION

1. CHARACTERIZATION OF SYNAPTOSOMAL O_2 UPTAKE

Before discussing the effects of anoxia and hypoxia on cerebral metabolism it is relevant first to consider the characteristics of synaptosomal O_2 uptake under normal conditions.

The synaptosomal limiting membrane encloses various cytoplasmic organelles including synaptic vesicles and one or more mitochondria (Jones & Bradford, 1971). Although there are many enzymes within the synaptosome which utilize molecular O_2, a vast excess of this O_2 is consumed by the intrasynaptosomal mitochondria. To what extent the synaptosomal limiting membrane influences the overall O_2 consumption of the enclosed mitochondria will be considered further.

One of the principal indices of mitochondrial integrity in vitro is the respiratory control index (RCI) which is defined as the ratio of the respiratory rate in the presence of ADP to that in its absence (Chance & Williams, 1955). For isolated cerebral mitochondria , the RCI can range from 2 to 11 depending upon the substrate employed (McIlwain & Bachelard, 1971). Within experimentally defined conditions, a high value for the RCI implies a tight coupling between respiration and oxidative phosphorylation, i.e. respiratory control. Verity (1972) demonstrated that the addition of ADP (0.16mM) stimulated glutamate-supported O_2 uptake by synaptosomes until the added ADP had been oxidatively phosphorylated to ATP, at which time the respiration reverted to the State 4 rate. The sharp distinction between State 3 and State 4 respiration here allowed calculation of the RCI as 4.9. These experiments were repeated by myself in order to establish my preparation of synaptosomes, from the rat cerebral cortex, as suitable for study with an O_2 electrode.

Although using a different preparation method (Bradford et al., 1975), I was unable to obtain a RCI value greater than 1.86 under identical incubation conditions and no sharp on-off transition between State 3 and State 4 respiration was obtained. These results were obtained with either glutamate (4 and 5mM) or succinate (4 and 5mM) although both were able to support ADP-stimulated O_2 uptake to a significant degree (see Table 6.2.3.).

There are two likely explanations for the discrepancy between the results described in Section 6.2 and those of Verity (1972). Firstly one must consider the possible contamination by free mitochondria in the two preparations. Because of the much higher specific activities of brain mitochondria to oxidize glutamate and succinate compared with synaptosomes (Clark & Nicklas, 1970; Lai & Clark, 1976), even a few per cent contamination of the synaptosomes by free mitochondria will contribute a disproportionate increase in O_2 uptake on the addition of ADP. Two pieces of evidence point to the conclusion that the synaptosomal fraction used by Verity (1972) was highly contaminated by free mitochondria, (1) the high activities of malate dehydrogenase and cytochrome c oxidase in the synaptosomal fraction when compared to the mitochondrial fraction as described in Verity (1972), and (2) the use of my crude mitochondrial fraction-containing myelin, synaptosomes and mitochondria - gave a mean value of 5.17 for the respiratory control index indicating the significant contribution made by the free mitochondria (see Table 6.2.9.(ii)). Booth & Clark (1978) subsequently reported a RCI value of 2.9 for their preparation of synaptosomes measured under identical conditions thus confirming my own results.

Another important point that is brought out by these experiments is the influence of the incubation medium. The experiments

described above were obtained using a high K^+ (100mM) medium and synaptosomes have already been found to behave differently depending upon the K^+ and Na^+ content of the incubation medium (Bradford, 1969). It is probable then that the use of Verity's medium, which resembles a typical incubation medium used for intracellular organelles, in fact biasses the responses towards those of the contaminating free mitochondria. For example, using a Krebs phosphate medium (high Na^+ and low K^+), the stimulation of O_2 uptake by glutamate and ADP was no longer found, similarly with glucose and ADP, see Tables 6.2.10. and 6.2.11. (confirmed by Booth & Clark, 1978). The rate of O_2 uptake tended to be higher in the Na^+ medium.

Further experiments were performed to establish the differences and similarities between synaptosomal and mitochondrial respiration. The typical responses of mitochondria include the inhibition of ADP-stimulated respiration by oligomycin which inhibits the mitochondrial ATPase involved in oxidative phosphorylation and the uncoupling of respiration from ATP synthesis by the addition of an uncoupling agent such as 2,4-dinitrophenol (DNP). Using the high K^+ medium of Verity (1972), the addition of 18μM oligomycin completely inhibited the ADP-stimulated synaptosomal O_2 uptake in the presence of 4mM glutamate and immediately reduced the rate to that in the presence of glutamate alone , see Chapter 2, Section 6.2.5. The further addition of ADP (0.16mM) failed to overcome this block in contrast to the addition of 100μM DNP which produced a significant increase in the rate of O_2 uptake, see Table 6.2.5.

As the behaviour of synaptosomes, with reference to their O_2 consumption, reflects the presence of the intrasynaptosomal mitochondria, what is the extent of the influence of the synaptosomal limiting membrane? The low values obtained for the RCI and the less well-defined

transition between State 3 and State 4 respiration demonstrated by the synaptosomal suspension may have been due to an inadequate rate of ADP translocation across the synaptosomal limiting membrane. In order to test this hypothesis, synaptosomes were subjected to various degrees of osmotic shock with the intention of rendering the outer limiting membrane leaky. During the preparative procedures, synaptosomes obtained from the sucrose density gradient are normally diluted 2-fold with H_2O to restore isotonic conditions (Gray & Whittaker, 1962). For these experiments, the synaptosomes were diluted up to 8-fold and a few samples were frozen and thawed before measuring O_2 uptake. As can be seen in Table 6.2.6. and Figure 6.2.6., hypotonic conditions reduce the rate of glutamate-supported O_2 uptake and significantly decrease the effectiveness of ADP and DNP to stimulate respiration. Translocation of ADP across the synaptosomal limiting membrane does not therefore appear to be the limiting factor in the response of synaptosomes to exogenous ADP. Bradford (1969) had similarly demonstrated that hypotonicity reduces the rate of O_2 uptake although on this occasion the synaptosomes were incubated in a Krebs phosphate medium with 10mM glucose.

The limiting membrane of the synaptosome has been shown to possess a Na^+, K^+-ATPase (Bradford et al., 1966; Ling & Abdel-Latif, 1968). Verity (1972) found a large stimulation of synaptosomal O_2 uptake due to Na^+ and interpreted this as being due to an increased generation of ADP by the Na^+, K^+-ATPase. Extrusion of Na^+ thus elevates ADP levels which in turn stimulates respiration. The results expressed in Table 6.2.7. - although few in number - tend to support these observations since 40mM Na^+ stimulated O_2 uptake in the presence of glutamate and ADP, and this stimulation was blocked by the addition of 180µM ouabain.

Having established the influence of the synaptosome limiting membrane in distinguishing synaptosomal from mitochondrial O_2 uptake, and the composition of the medium on the overall rate of O_2 uptake, let us now consider the effects of near-physiological conditions. The following results have been obtained using Krebs phosphate medium, pH 7.8 (Bradford et al., 1975), at 30°C and saturated with air to give an O_2 concentration of approximately 220μM (21% or 158.8mmHg). In the experiments of Verity (1972) bovine serum albumin (BSA) had been added to the synaptosomal suspension to produce optimal respiratory conditions and a zero Ca^{2+} concentration was used to prevent activation of phospholipases. The use of zero Ca^{2+} is in contrast to a study of brain phospholipases A_1 and A_2 by Cooper & Webster (1970) who demonstrated that neither enzyme required Ca^{2+} for activity. To establish the requirement for BSA, two series of experiments were performed in the absence and presence of BSA (3.33mg/ml). In both series, ADP failed to stimulate glucose-supported synaptosomal O_2 uptake and the addition of DNP in the absence of BSA significantly increased the rate of O_2 uptake when compared to the rate in the presence of BSA suggesting that BSA is in some way preventing the full uncoupling process, see Table 6.2.11.

For the purpose of this discussion, the "control" condition has been taken as the rate of synaptosomal O_2 uptake in the absence of exogenous substrate with the synaptosomal pellet having been kept on ice until required. As has been described in Chapter 2, Section 7.3.1., variations in the dimensions of the pestle and tube used to prepare the initial homogenate have marked effects on the calculated rate of synaptosomal O_2 uptake. This can range from 24 to 52μmoles O_2/hr/100 mg protein and for this reason I shall avoid the use of absolute values and concentrate on the direction of responses in synaptosomes prepared under identical conditions.

Figure 7.2.1.(a) shows that the addition of glucose (10mM) significantly stimulated the rate of O_2 uptake whereas the addition of BSA (3.33mg/ml) was without effect. When BSA was however added in the presence of glucose there was a significant stimulation of synaptosomal O_2 uptake, presumably due to the presence of glucose. The presence of an exogenous substrate such as glucose would be expected to increase O_2 consumption (Bradford, 1969) and we have already observed that BSA at this concentration is without effect (Table 6.2.11.). It is interesting to observe that all these responses are present regardless of the control rate, i.e. when using different homogenization conditions. Figures 7.3.1.(i) and 7.3.1.(ii) indicate that the responses are qualitatively but not quantitatively similar.

The response to BSA was found to be influenced in a concentration-dependent manner. For example, in another series of experiments increasing the concentration of BSA from 3.33 to 13.33mg/ml produced a significant increase in the rate of synaptosomal O_2 uptake, see Figure 8.2.1.(i). These results can be expressed in terms of the ratio of synaptosomal protein to the amount of BSA, for a BSA concentration of 3.33mg/ml gives a tissue:BSA ratio of 5.8 : 10 and at 13.33mg/ml the ratio becomes 1.45 : 10. Therefore a ratio of 5.8 : 10 produces a significant decrease here in the rate of O_2 uptake whereas a ratio of 1.45 : 10 significantly increases O_2 uptake by synaptosomes.

How then does BSA exert its effect? Pressman & Lardy (1956) provided evidence that free fatty acids (FFA) were the agents in microsomes responsible for activation of latent mitochondrial ATPase and respiration. Equilibrium dialysis studies on BSA and fatty acids had already shown the strong affinity of long chain fatty acid anions for BSA which decreased with decreasing chain length (Teresi & Luck,

1952). BSA contains six high-energy binding sties for long chain FFA and a large number of weaker binding sites (Spector et al., 1969). The addition of BSA to mitochondria in vitro was found to prevent the decay of DNP-stimulated ATPase activity with the effect being fairly specific since ovalbumin, lactalbumin and γ-globulin were ineffective (Chefurka, 1966). BSA is not taken up to any significant extent by mitochondrial membranes and is therefore unlikely to act by repairing the mitochondrial membrane (Chan & Higgins, 1978).

Ageing (liver) mitochondria are characterized by an accumulation of FFAs such as palmitic, stearic, oleic, linoleic and arachidonic acids. 18-carbon and long unsaturated fatty acids were more effective than their corresponding saturated acids in inhibiting DNP-stimulated ATPase activity (Borst et al., 1962; Chefurka & Dumas, 1966) and oleic acid is generally considered as the major uncoupling agent in mitochondria (Borst et al., 1962). As shown in Table 10.2.3.(b), long-chain unsaturated FFAs make up the largest proportion of the total FFAs obtained after a 10 min period of incubating synaptosomes.

Although it would be expected that FFAs, acting as uncouplers, would stimulate respiration the opposite effect is more often observed. Exogenous oleic acid inhibited the respiratory activity of brain mitochondria (Lazarewicz et al., 1972; Lochner et al., 1976) with the decrease in ATP synthesis being greater than the decline in O_2 uptake (Kuwashima et al., 1976). FFAs might be acting by damaging the mitochondrial membrane and allowing the leakage of respiratory cofactors. Replacing these cofactors, e.g. NAD^+, CoA and cytochrome c, restored mitochondrial oxidation (Bjorntorp et al., 1964) and restored mitochondrial O_2 uptake in the presence of FFAs (Lochner et al., 1976). Bjorntorp et al. (1964) demonstrated that mitochondrial oxidative activity in the presence of BSA proceeds at substantial rates, tightly

coupled to phosphorylation, with high P:O ratios and without ATPase stimulation, even at relatively high fatty acid concentrations, provided that sufficient BSA was present to bind the fatty acids.

It seems likely then that BSA in sufficient concentration increases synaptosomal respiratory activity by effectively removing free fatty acids in an analogous manner to its action on mitochondrial respiratory activity *in vitro*. An interesting point to add here regarding neuronal tissue is the observation of Aveldaño & Bazán (1974) who incubated isolated bovine retina in a fully oxygenated medium containing glucose. They found a 2-fold increase in the FFA pool size within 5-20 min of incubation with the FFAs being displaced into the medium by albumin. Approximately 18% of the total FFA were released into the medium at low albumin concentrations (0.1mg/ml) and up to 60% at a higher concentration of 20mg/ml although the total content of tissue plus medium was not significantly altered by the varying concentrations of albumin. Removal of FFAs from direct action on the tissue may be responsible for the protective nature of albumin in preserving the RCI of mitochondria *in vitro*.

Although both glucose and BSA are able to stimulate synaptosomal O_2 uptake, the characteristics of the responses are markedly different. Using Figure 8.2.1.(i) as an example, the control rate falls off slowly over the course of the experiment whereas the presence of glucose maintains the rate of O_2 uptake at a more constant rate over the approximate 20 min incubation time (see also Section 7.2.1.). Although BSA at high enough concentrations can stimulate synaptosomal O_2 uptake, it is unable to maintain this increased rate in the manner observed for glucose.

In conclusion, synaptosomal O_2 uptake shows many of the characteristics of mitochondrial respiration:

(i) when placed in a mitochondrial medium, synaptosomes respond to the addition of ADP by increasing O_2 consumption when supported by exogenous substrates such as glutamate and succinate. This response is not as distinct as found in mitochondria nor as marked as suggested by Verity (1972). These responses are also absent in a high Na^+ (Krebs phosphate) medium.

(ii) the presence of the synaptosomal limiting membrane does not limit the ADP response by hindering the translocation of ADP. Rendering the limiting membrane leaky does not improve the respiratory control index and increasing the degree of hypotonic shock reduces the O_2 consumption by synaptosomes.

(iii) synaptosomes behave like mitochondria in their response to oligomycin which blocks the ADP response in a high K^+ medium, and to an uncoupling agent such as 2,4-dinitrophenol which can overcome the oligomycin-block where ADP is ineffective.

(iv) in a high K^+ medium, Na^+ increases O_2 uptake presumably by stimulating the Na^+, K^+-ATPase located in the synaptosomal limiting membrane.

(v) in a normal Na^+ medium, synaptosomal O_2 uptake is stimulated by glucose in long-term experiments and in a different manner, is also stimulated by the presence of bovine serum albumin when present in sufficient amounts.

2. THE EFFECTS OF ANOXIA AND HYPOXIA ON CEREBRAL METABOLISM

2.1. Introduction

It has been well established that although even relatively moderate degrees of arterial hypoxia lead to changes in cerebral function, metabolism and blood flow, P_{AO_2} in experimental animals can be lowered to 20-25mmHg without causing a clear perturbation of tissue concentrations of ATP, ADP and AMP (Duffy et al., 1972; MacMillan & Siesjö, 1972; Bachelard et al., 1974). Of particular relevance to this discussion are questions such as:

(a) Is there a critical P_{O_2} with relevance to metabolism in the nerve-ending?

(b) Why does neuronal activity cease before energy failure in hypoxia?

2.2. Is there a critical P_{O_2} with relevance to metabolism in the nerve-ending?

The metabolic properties of synaptosomes have already been described (Chapter 1, Section 15.4.) along with details of synaptosomal O_2 uptake under normal conditions (Chapter 3, Section 1). Now I shall consider the influence of anoxia and hypoxia in particular on synaptosomal O_2 uptake and metabolism in the hope of providing further information which might help in answering the questions raised above.

Firstly one must consider whether or not synaptosomal O_2 consumption is influenced by the concentration of O_2 in the surrounding medium. From experiments performed to monitor synaptosomal O_2 uptake after the synaptosomes have been placed in air-saturated Krebs phosphate medium at 30°C, one can see that the rate of O_2 uptake remains fairly constant until the O_2 concentration in the medium has fallen to approximately 30μM (approx. 20mmHg), see Figures 7.2.1.(a) and 8.2.1.(i). Further experiments were performed using initial O_2

concentrations at values above and below 30μM, see Figures 7.2.2.(a)-(e). Here one can see that synaptosomes behave in conditions of reduced P_{O_2} in a similar manner as in normoxic conditions in responding to the presence of glucose and BSA by significantly increasing their rate of O_2 uptake. Interestingly, if these results are combined as in Figure 7.2.3.(i), the response to BSA (3.33mg/ml) appears to dominate at O_2 concentrations below 70μM (approx. 50mmHg). The effect can be resolved simply by consideration of the ratio of tissue protein to BSA since smaller tissue samples were used in the reduced O_2 experiments thus reducing the ratio by half and therefore making the BSA apparently more effective, see previous Section (1).

The independent effects of glucose and BSA can be seen more clearly (Figures 7.2.3.(ii) and 8.2.1.(ii)) suggesting an additive response when the two are present together. Here also is evidence for a critical O_2 concentration at approximately 4μM above which O_2 consumption by synaptosomes appeared to be relatively independent of the P_{O_2}. The value of the critical P_{O_2} for synaptosomal O_2 uptake is independent of initial P_{O_2} values and to a lesser extent is independent of the initial rate of O_2 consumption. The arterial P_{O_2} value for alterations in the EEG of whole animals is approximately 30mmHg (approx. 42μM) which, even accounting for its being an overestimate of actual tissue P_{O_2}, is considerably higher than the value obtained here for alterations in synaptosomal O_2 consumption.

Much data already exists for mitochondria <u>in vitro</u> giving a critical P_{O_2} of less than 5mmHg (approx. 7μM), (Jöbsis, 1972). Various definitions have been given to the term "critical P_{O_2}" but the one most favoured and the one used here is the transition point where the demand for O_2 changes from zero order (O_2 independent) to 1st order (O_2 dependent), (Wise, 1951). When determining the critical P_{O_2}

of isolated cells it is important that the cells are well dispersed as the critical P_{O_2} will appear higher when they are clumped together and diffusion of O_2 is restricted (Jöbsis, 1972).

Kovalenko & Grinberg (1972) demonstrated that the respiratory activity and the critical O_2 concentration of rat brain or liver mitochondria were independent of each other. For example, ADP increased both mitochondrial respiration and the critical P_{O_2} whereas DNP increased respiration but not the critical P_{O_2}. In intact mitochondria, the apparent K_m for O_2 is dependent on the energy state although reports in the literature give this influence in contradictory directions (Chance, 1957; Petersen et al., 1974).

An approximation of the K_m for O_2 in synaptosomal O_2 consumption can be calculated as 4μM when K_m is determined as the substrate concentration at half maximum velocity in glucose-supported respiration (Figures 8.2.1.(i) and (ii)). For comparison, the apparent K_m for O_2 for rat brain mitochondrial respiration is 0.1μM (Clark et al., 1976).

Although it has been shown that synaptosomes behave in a similar manner whether placed in normoxic or hypoxic conditions, at least at a P_{O_2} greater than the critical O_2 concentration, there are some slight but intriging differences. Figure 8.2.2. compares the rate of synaptosomal O_2 uptake at P_{O_2} 150 and 10mmHg with the rate at 10mmHg after having started the incubation at 150mmHg (normoxia). The rates to be examined are those in Krebs phosphate alone, with glucose (10mM) and with BSA at the low tissue to BSA ratio. The rate of synaptosomal O_2 consumption in the presence of BSA is significantly higher when the tissue is placed in hypoxic conditions than when it was originally placed in normoxic conditions. It is likely that during the 15-20 minutes taken by the synaptosomes to reduce the P_{O_2} from 150 to 10mmHg, free

fatty acids (FFA) were released in excess of the ability of BSA to bind them thus inhibiting respiratory activity (Bjorntorp et al., 1964).

Experiments designed to measure the accumulation and distribution of FFAs after 10 min incubation periods failed to show any significant differences between normoxic incubation of synaptosomes and hypoxic or anoxic incubations (Tables 10.2.3.(a) and (b)). These results are surprising because incubation of brain tissue has been reported to increase the yield of FFAs (Lunt & Rowe, 1968; Aveldaño & Bazán, 1974), especially in ischaemic-anoxia (Bazán & Cummings, 1969; Bazán, 1970). It is probable that the experimental procedure could be improved here to resolve these conflicting reports, see Chapter 2, Section 10.2.3. Incubating synaptosomes at P_{O_2} 100mmHg (approx. 140μM) for 30 min at 30°C produced no change in the distribution of free or occluded lactate dehydrogenase activity (Table 10.2.1.). If there had been any major disruption in the structure of the synaptosomal limiting membrane then one would have expected to find an increase in free lactate dehydrogenase activity (Marchbanks, 1967).

In the absence of exogenous substrate, synaptosomes might depend upon endogenous amino acids such as glutamate, GABA and aspartate to act as respiratory substrates. When placed in an incubation medium, synaptosomes rapidly lose glutamate, GABA and aspartate to the medium and these amino acids can be taken back up on incubation at room temperature or 37°C but not at ice temperature (Bradford et al., 1975). From this observation, one would expect that the rate of synaptosomal O_2 uptake could be maintained in the absence of exogenous substrate although from Figure 8.2.2. one can see that the rate of O_2 consumption has fallen quite markedly and is not significantly different from the rate found when the initial P_{O_2} is low. For the latter condition the tissue has been taken straight from on ice and there would have been no

chance to re-accumulate these lost amino acids.

Although not statistically significant, the presence of glucose during the 15-20 minutes that it takes to reduce the P_{O_2} from 150 to 10mmHg does allow increased respiratory activity in comparison to tissue placed in a medium already at a low P_{O_2}. This result would be expected as glucose is known to support synaptosomal O_2 uptake for periods as long as 3-4 hr (Bradford et al., 1975).

It is because of the influence of time which effectively allows "pre-incubation" to occur before reaching hypoxic conditions that these results cannot be used in the determination of a true K_m value for O_2 in synaptosomal respiration.

In order to look more closely at the influence of incubation times on determining the rate of synaptosomal O_2 uptake in hypoxic conditions, the synaptosomes were pre-incubated for 35 min at 30°C before being placed with the O_2 electrode. The pre-incubations were performed in the same tubes as used to store the tissue on ice and the water bath was used either in the non-shaking or the shaking mode. Two distinct patterns of responses emerged depending upon the type of pre-incubation. When synaptosomes were pre-incubated in Krebs phosphate medium alone and then glucose (10mM) or BSA (3.33mg/ml) were added in the O_2 electrode chamber under hypoxic conditions ($P_{O_2} \leq$ 10mmHg, 14μM), all the responses found without the pre-incubation were present if the shaking water bath had been used (Table 8.2.3.). The only differences here were that the rates of synaptosomal O_2 uptake were reduced compared to the non-incubated tissue.

In contrast, pre-incubation in a non-shaking water bath prevented the expected stimulation of O_2 uptake or the subsequent addition of glucose or BSA (Table 8.2.4.). Under these conditions the

control rate (Krebs) was no longer significantly reduced but was no different from the control rate in the absence of any pre-incubation. Glucose failed to stimulate O_2 consumption and BSA produced a significant reduction in the rate of synaptosomal O_2 uptake. This latter result cannot be considered as the consequence of accumulated FFA in excess to the binding capacity of the BSA as the result should then be identical or greater than the control rate under the same conditions.

To increase the degree of comparison between these pre-incubation experiments and the "pre-incubation" which occurs when the synaptosomes are placed in air-saturated media, glucose and BSA were added to the resuspended synaptosomal pellets during the pre-incubation time of 35 min. The use of shaking pre-incubation allowed the presence of glucose throughout the pre-incubation time to produce a more highly significant stimulation of synaptosomal O_2 uptake thus indicating the importance of exogenous glucose as a respiratory substrate for synaptosomes. The presence of BSA during the pre-incubation did not however afford any degree of protection. The presence of glucose or BSA during the non-shaking pre-incubation produced identical results to those in their absence - even the presence of glucose could not better the rate of O_2 consumption.

These results have been combined in Figure 8.2.5. which makes it clear that the pre-incubation procedure used has not helped to clarify the original question and this may simply be due to an incorrect matching of pre-incubation time and uncertainty over the degree of oxygenation during the shaking and non-shaking pre-incubations.

One point does stand out here and adds further confirmation to the idea that glucose and BSA are acting independently. Whether glucose and BSA increase, decrease, or have no effect on the rate of synapto-

somal O_2 uptake, when they are present together the response can be predicted by addition of the individual responses (Tables 8.2.3. and 8.2.4.).

Why the mechanics of the pre-incubation procedure should have such a marked influence on the behaviour of the synaptosomes is uncertain. One might expect different degrees of oxygenation depending upon movement of the suspension which would allow for better mixing, although it seems reasonable to expect that the results would only be quantitatively different and not qualitatively so. Even so the influence of mixing is probably minimal due to the small volumes used (250µl). Similarly although both forms of pre-incubation in Krebs phosphate medium significantly increased the proportion of free to occluded lactate dehydrogenase activity when compared to tissue kept on ice, there was no difference between the two forms of pre-incubation (Tables 8.2.6.(i) and (ii)). The presence of glucose during the pre-incubations resulted in a further increase in the proportion of free lactate dehydrogenase activity.

One could speculate here that the use of non-shaking pre-incubation before hypoxic incubation is very similar to the response of mitochondria following recirculation after incomplete ischaemia. Mitochondria isolated from brain after incomplete ischaemia show continued deterioration as opposed to extensive functional recovery following recirculation after complete ischaemia (Rehncrona et al., 1979). Synaptosomes following non-shaking pre-incubation fail to respond in the "normal" manner and in so doing have become less flexible in behaviour. It does appear somewhat contradictory though that an equivalence can be drawn between incomplete ischaemia and non-shaking pre-incubation, and complete ischaemia with shaking pre-incubation.

Similar results have been obtained when the pre-incubated synaptosomes were subsequently placed in air-saturated media in contrast to the hypoxic conditions described above. Here one can see that although all the rates are lower than the control rate for non-pre-incubated synaptosomes, the glucose response is maintained by the shaking pre-incubation conditions but is lost by the non-shaking pre-incubation procedure (Table 8.2.5.(i)). These responses are therefore due primarily to the influence of the pre-incubation and not to the subsequent incubation in hypoxic conditions.

As already described, the pre-incubation in a non-shaking water bath has the most profound effect on synaptosomal O_2 consumption. Bradford (1969) found that 20mM-NaH_2PO_4 was necessary for maximum synthesis of ATP and PCr. A reduction in the concentration of NaH_2PO_4 to 39% of normal, whilst not affecting the control rate of synaptosomal O_2 uptake in hypoxia did prevent the stimulation normally seen in the presence of glucose or BSA (Table 9.2.2.). Of greater significance was the effect of a non-shaking pre-incubation which after only 5 min produced a highly significant fall in the rate of synaptosomal O_2 uptake (Table 9.2.2.). When the pre-incubation time was extended from 5 to 35 min, a further significant reduction in O_2 consumption occurred suggesting that the synthesis of high-energy phosphate bonds had been drastically reduced and the energy-dependent reactions within the synaptosomes would be consequently compromised. Such a dramatic response - a 66% fall in O_2 consumption - was not observed after pre-incubation in normal Krebs phosphate medium.

Do these results tell us anything about predicting nerve function *in vivo* when subjected to hypoxia? Firstly it is important to remember that synaptosomes are in fact artefacts and not found in the brain *in vivo*. Therefore they lack a vast array of both neuronal and

extra-neuronal influences. In the context of these pre-incubation experiments just described, it has been suggested that synaptosomes should be pre-incubated for 15 min before using them in transport studies (Marchbanks, 1975). Although prewarming synaptosomes at 30°C for 20 min prior to use has been found not to improve the observed decrease in measured L-glutamate uptake of synaptosomes stored in 0.32M ice-cold sucrose for periods longer than 50 min (Wheeler, 1978).

It is quite obvious from the results in Tables 8.2.3. and 8.2.4. that a shaking water bath should always be used, preferably with an adequate source of respiratory substrate such as glucose in order to obtain a preparation of synaptosomes showing maximal respiratory activity. Is glucose all that is required? Well it has been suggested that glutamine in CSF is likely to make an essential contribution in vivo as a substrate for nerve-endings, (see Chapter 1, Section 12.4.) and therefore it should be included in incubation fluids as a standard component (Bradford & Ward, 1975). This has support from the work of Phizackerley & Fixter (1973) who measured O_2 uptake by rat brain slices. These authors found that increasing the duration of anaerobic (N_2) pre-incubation in an incubation medium lacking glucose progressively impaired the subsequent incubation in a glucose medium. This effect was temperature sensitive - not seen at < 20°C - and was not due to increased acidity. A protective effect was observed when using unsliced hemispheres and the protective agents were found to be L-glutamine and L-aspartate. The anaerobic loss of glutamine and aspartate was decreased when glucose was added to the medium. This finding implies that the normal respiratory metabolism of glucose by brain is dependent upon the maintenance of adequate tissue concentrations of these amino acids and that their loss is an important factor in the reduced ability to oxidize glucose which results from anoxia in vitro. Since the principal source of glutamine is likely to be the CSF (Bradford & Ward, 1975) and

from the conversion of glutamate to glutamine by glutamine synthetase localised in both glia and the neuronal perikarya (Weiler et al., 1979), then one must expect that synaptosomal behaviour under conditions of stress, e.g. anoxia and hypoxia, will not be completely comparable to the behaviour of intact nerve-endings. This however does not detract from the usefulness of studying nerve-endings in isolation since comparison with brain slice experiments allows one to pinpoint the likely localization and cause of the metabolic defect.

In addition to the likely involvement of glutamine and aspartate deficiencies in impairing synaptosomal O_2 consumption in the absence of glucose, there is also the possibility that a disturbed ionic balance may be responsible as synaptosomes fail to accumulate K^+ to the same extent as when glucose is in excess (Bradford et al., 1975). Preincubation of rat cerebral cortex slices with a high-speed brain homogenate supernatant prevented the anoxic impairment of subsequent respiration (Patel & Fixter, 1975), an effect that was suggested to be due to K^+. Both anoxia and hypoxia are known to cause the release of K^+ from the intracellular compartments (Hansen, 1977; Bourke et al., 1978), and Patel & Fixter (1975) found that a K^+ concentration in slices of 20μequiv./g final wet weight was required to maintain respiratory activity.

Going up the scale further from brain slice experiments to whole animal experiments, are there any reports of improved functioning after a period of O_2 lack? Here one needs to consider the impact of a sudden O_2 deficit rather than the well known phenomenon of the ability to acclimatize to high altitude conditions. Dahl & Balfour (1964) discovered that pre-exposure of rats to anoxia increased their survival time on re-exposure. This improvement in survival time was not due to increased reserves of ATP but rather to pyruvate. The increase in

lactate during the first exposure to anoxia is rapidly metabolised to pyruvate during the recovery period and ATP returns to normal. If the repeat occurs before pyruvate has been dissipated, glycolysis can proceed at a faster rate due to the greater oxidation of NADH by the accumulated pyruvate. Because energy is produced at a greater rate, ATP falls more slowly and the rat can live for 90 sec instead of only 60 sec - not a vast improvement in terms of time but still important in indicating the important matching of anaerobic to aerobic glycolysis.

So far I have shown that synaptosomes respond in a similar manner to the addition of glucose or BSA regardless of whether they are initially placed in media of high or low O_2 content, provided that they are taken straight from storage on ice (Figures 8.2.1.(i) and 8.2.1.(ii)). During the course of an experiment one finds that the basal level of O_2 consumption gradually declines and this decline cannot be improved upon by pre-incubating the tissue before measuring the rate of O_2 uptake. The presence of glucose during the incubation of synaptosomes in the O_2 electrode chamber allows for increased rates of O_2 uptake, and more importantly O_2 consumption can be maintained at a constant rate in contrast to respiration depending upon endogenous substrates. However if adequate respiratory rates are to be obtained after a period of pre-incubation, glucose must be present during the shaking pre-incubation process in order to build up and maintain levels of ATP and PCr which will be required to keep the isolated nerve-endings in a viable condition.

2.3. Why does neuronal activity cease before energy failure in hypoxia?

As already described in the introduction, synaptic transmission is more vulnerable to O_2 lack than is axonal conduction (Dolivo, 1974) and the functional/behavioural alterations observed in hypoxia are due principally to a failure in transmission rather than an

energy failure (Siesjö et al., 1974). What then are the features of the synaptic transmission process and which of these components are likely to be affected by an O_2 lack? Although it is difficult to discuss the following components of the stimulus-secretion coupling process in isolation, they can be separated as: membrane integrity; mitochondrial function and neurotransmitter synthesis and metabolism. One must remember that for the purpose of this discussion the assumption is that there is no disruption of the energy-supplying reactions in hypoxia.

There seems to be very little information covering aspects of nerve function in hypoxia, except for the measurement of intermediates in the energy producing systems and neurotransmitter synthesis and metabolism. Information concerning membrane integrity and mitochondrial function comes from extrapolation of data from experiments involving anoxic-ischaemic brains, and from my own experiments.

The structure of synaptosomes remains very well preserved after incubation in tissue culture media for as long as 24 hr, even though their metabolic activity ceased after 10 hr or so (Bradford et al., 1975). Morphological integrity may therefore not be a good index of biochemical viability. However ultrastructural changes have been found in conditions of O_2 lack (see Chapter 1, Section 10). Of particular interest is the observation that when the isolated retina is deprived of O_2 for 10 min, one finds a loss and clumping of synaptic vesicles (Webster & Ames, 1965). Similarly, microscopic examination of an ischaemic cat brain showed clumping of vesicles away from the synaptic cleft in approximately 10% of synaptic endings and a 2-fold increase in the number of presynaptic profiles devoid of vesicles (Williams & Grossman, 1970). It would be interesting to know whether identical changes are found in hypoxic brains since this

could provide a clue to the neuronal dysfunction which occurs in hypoxia.

Evidence for changes in synaptic membrane composition is lacking in terms of mild O_2 lack although there is plenty of information for the more drastic conditions of anoxia and ischaemia. In particular, interest has been focussed on the free fatty acid (FFA) content of cerebral tissues. In normal brain, fatty acids occur primarily as constituents of phospholipids and glycolipids. FFAs are present only in very low concentrations (Rowe, 1964; Lunt & Rowe, 1968) and have been estimated at 4.5mg FFA/g protein in synaptosomes (Price & Rowe, 1972). Although Eichberg et al. (1964) found no loss of phospholipid during the subfractionation of brain, Lunt & Rowe (1968) measured an increase in FFAs on subfractionation of brain slices. Similarly FFAs were found to increase on incubation (Lunt & Rowe, 1968; Aveldaño & Bazán, 1974). Tables 10.2.3.(a) and (b) show the results of incubating synaptosomes at 30°C for 10 min in Krebs phosphate medium containing glucose (10mM). Three conditions were used: normoxia (P_{O_2} 150mmHg, approx.), hypoxia (P_{O_2} < 10mmHg) and anoxia (P_{O_2} zero). The measurable FFAs were palmitic, stearic, oleic, linoleic and arachidonic acids plus three unidentified peaks which ran between linoleic and arachidonic acid on the GLC. It was surprising to find that hypoxia and in particular anoxia showed no difference in both the quantity of FFAs and the distribution of the different fatty acids between the separated tissue and incubation medium.

In the ischaemic brain (decapitation) there is an immediate rapid accumulation of FFAs with the enlargement of the total pool due to production of arachidonic, oleic, stearic and palmitic acids (Bazán, 1970). Similarly both mitochondria from ischaemic brains and those made anoxic in vitro show a similar accumulation of FFAs

(Lazarewicz et al., 1972; Markelonis & Garbus, 1975). Either nerve-endings are not the source of FFAs accumulating in anoxic-ischaemic brains or the experiments described in Chapter 2, Section 10.2.3. require improvement to reduce the errors and so provide confirmation for the results reported here.

Whether or not nervous tissue releases FFAs under conditions of mild hypoxia is therefore uncertain at the present time. With the widely accepted idea that BSA is added to mitochondria in vitro to preserve the RCI by binding FFAs, how does this fit in with the results on synaptosomal O_2 consumption? The suggested influence of FFAs on mitochondrial respiration acting either as uncoupling agents (Borst et al., 1962) or by allowing the leakage of respiratory cofactors (Bjorntorp et al., 1964; Lochner et al., 1976) has already been discussed in the previous Section.

BSA functions in a concentration-dependent manner (Figure 8.2.1.(i)) and is equally effective when used in a purer form which is essentially fatty acid free (Table 9.2.1.). This result suggests that the BSA normally used is not contributing fatty acids to the synaptosomal suspension which could in turn uncouple respiration. Neither does a solution of BSA in Krebs phosphate medium (3.33mg/ml) show a greater loss of O_2 from the O_2 electrode chamber compared to the Krebs phosphate medium alone. On intriguing and as yet unconfirmed possibility is that BSA could be exerting its effect by binding Ca^{2+}. BSA has a strong affinity for ions and has been found to have a profound effect on cardiac function, an effect suspected of being caused by the binding of Ca^{2+} (M.J. Parry, personal communication).

To support this possibility is the observation that although blood plasma contains 2.5-3mM-Ca^{2+}, only some 30% of this is free and unassociated with protein (McIlwain & Bachelard, 1971). It should be

noted that in Krebs phosphate medium buffered with 20mM phosphate, a large proportion (65%) of the 0.75mM calcium is present as the un-ionized soluble monohydrogen phosphate (Dodd et al., 1971). However a calcium concentration of 0.75mM has been found to be optimal in maintaining isolated cerebral tissues with adequate Na^+ and K^+ (McIlwain & Bachelard, 1971).

When synaptosomes were resuspended in a Ca^{2+}-free medium with EGTA and then placed in a hypoxia medium of the same composition, the rate of O_2 uptake was significantly increased (Table 9.2.1.) and the shape of the O_2 consumption curve appeared identical to the BSA curves (Figure 9.2.1.). Decreasing the Ca^{2+} concentration of the medium down to zero levels is well known to stimulate respiration and glycolysis (McIlwain, 1952) even though the presence of Ca^{2+} in concentrations of 1-2mM is required to maintain long-term respiration (Dickens & Greville, 1935). Bradford et al. (1973) found that the absence of Ca^{2+} greatly accelerated respiratory (by 30% of normal) and glycolytic rates of synaptosomes. The stimulated respiration has already been noted (Table 9.2.1.) and increased glycolysis is shown in Table 10.2.2.(ii).

Synaptosomes were incubated at 30°C for 10 min in hypoxic conditions using a Ca^{2+}-free medium with EGTA plus glucose. At the end of the incubation period, metabolism was stopped and lactic acid was extracted by the use of ice-cold perchloric acid. The absence of Ca^{2+} produced a significant increase in the production of lactic acid over the 10 min incubation period compared to the condition of hypoxia alone. The absence of Ca^{2+} has therefore been shown to increase both respiration and glycolysis in synaptosomes incubated under hypoxic conditions.

Lactate formation from glucose by cortical slices also occurs at a high rate in a Ca^{2+}-free bicarbonate medium and this rate is decreased by increasing calcium. These results led Dodd et al. (1971)

to suggest that Ca^{2+} ions could be decreasing glucose uptake by interacting with phosphate groups (see above) in the membrane that are involved in glucose transport.

The rate of aerobic glycolysis is increased when Ca^{2+} is omitted from the medium (Dickens & Greville, 1935; Lazarewicz et al., 1978) producing a fall in PCr levels (McIlwain, 1952). The control of aerobic glycolysis in cerebral tissues shows many signs of being intricately connected with the functional activity of the tissue. The concentration of free Ca^{2+} outside and within neurones is an important determinant of the functioning of the nervous system (Goddard & Robinson, 1976) and because Ca^{2+} stabilizes plasma membranes (Baker, 1972), its omission is likely to increase the permeability of the synaptosomal plasma membrane to cations. For example, spontaneous neurotransmitter release from synaptosomes can be augmented when the external Ca^{2+} concentration is zero by interfering with mitochondrial sequestration of Ca^{2+} (Silbergeld, 1977).

However with regard to hypoxia, there is no evidence of a changed distribution of Ca^{2+} and an altered Na^{+} distribution on which both Ca^{2+} influx and efflux depends (Blaustein & Weismann, 1970).

Further possible changes in synaptosomal membrane structure will now be considered. Bazán (1970) suggested that the likely source of FFAs liberated after decapitation were phospholipids. The brain is a lipid-rich organ containing up to 60% lipid on a dry-weight basis with phosphoglycerides rich in C20 and C22 polyunsaturated acids derived from the dietary essential fatty acids, linoleic and γ-linolenic (Hassam & Crawford, 1976). It is well known that the proper structural composition of lipids is a prerequisite for the functional integrity of membranes. Currently there is no evidence that nerve-endings in vivo are the source of the FFAs released during ischaemic-anoxia but the

possible consequences of such a loss are considerable and will be discussed here.

Anoxia has been reported to inhibit lipid synthesis from labelled acetate and glucose (Kosow et al., 1966). Under normal conditions brain lipid composition depends on a balance between the deacylation via phospholipase A_2 and reacylation via the acetyl-transferase of membrane phosphoglycerides with the latter reaction proceeding at a greater rate in brain tissue (Sun et al., 1979). The reacylation reaction in synaptosomes includes an initial energy-dependent activation of the fatty acids to the acyl-CoA derivatives and therefore requires ATP, Mg^{2+} and CoA (Corbin & Sun, 1978). In anoxia and ischaemia, [ATP] is reduced (Drewes & Gilboe, 1973a) and the loss of CoA has been implicated in reduced mitochondrial function (Bjorntorp et al., 1964; Lochner et al., 1976) therefore deacylation is likely to predominate in both anoxia and ischaemia.

It is interesting that the injection of lysophosphatidylserine into mice led to a fall in brain energy metabolism whereas both lysophosphatidylcholine and lysophosphatidylethanolamine were without effect (Bruni et al., 1979). Another consequence of phospholipase activity concerns lysophosphatidylcholine. The hydrolysis of membrane-bound phosphatidylinositol in rat liver microsomal fractions by the soluble phosphatidylinositol phosphodiesterase from rat brain was markedly stimulated by oleic and arachidonic acids (Irvine et al., 1979). This stimulation was totally suppressed by lysophosphatidylcholine. Phosphoinositides are uniquely enriched in arachidonic acid but whether or not arachidonic acid regulates cerebral phosphatidylinositol turnover is unknown. Arachidonic acid is one of the long-chain unsaturated fatty acids released during anoxia and ischaemia (Bazán, 1970) and because phosphatidylinositol has been implicated in synaptic transmission

(Aveldaño & Bazán, 1975; Corbin & Sun, 1978), its selective degradation may be related to the high vulnerability of the mammalian brain to O_2 deprivation.

There is evidence for the uptake of labelled fatty acids by guinea-pig synaptosomes with a relatively high rate of de novo synthesis of phosphatidylinositol at the nerve terminal compared with the whole brain (Baker et al., 1976).

The release of arachidonic acid by phospholipase A may be related to the regulation of arachidonic acid for prostaglandin biosynthesis. Arachidonic acid is the precursor of the predominant (dienoic) type of prostaglandins e.g. PGE_2 and $PGF_{2\alpha}$ (see Chapter 1, Section 11.2.). The enzyme cyclooxygenase which converts arachidonic acid to prostaglandin endoperoxides requires O_2 in a ratio of 2:1, O_2: arachidonate, therefore under normal conditions the supply of arachidonic acid is the limiting factor. Since the re-incorporation of arachidonic acid into lipids is reduced in anoxia and ischaemia, then decreased O_2 leads to increased prostaglandin synthesis whereas total O_2 lack will inhibit prostaglandin synthesis. The apparent K_m for O_2 is 5μM for the formation of prostaglandins by vesicular gland cyclooxygenase (Lands et al., 1978) which suggests that hypoxic conditions will not appreciably lower prostaglandin synthesis until the O_2 concentration falls to less than 5μM.

The increased production of prostaglandins which is likely to occur in hypoxia will have a profound affect on the outcome of a hypoxic insult. For example, the release of free arachidonic acid and the formation of $PGF_{2\alpha}$ in ischaemia is greater in brain cortex than in cerebellum (Bosisio et al., 1976) an affect possibly related to the selective vulnerability of the cortex (see Chapter 1, Section 11.4.) and similarly one might expect to find differential production of prosta-

cyclin and thromboxane A_2. Prostacyclin is a powerful vasodilator and an extremely potent inhibitor of platelet aggregation in contrast to the opposing actions of thromboxane A_2. Therefore the potential activation of thromboxane A_2 in hypoxia will be one of the deciding factors in the clinical outcome of O_2 deprivation since the occurrence of ischaemia appears to be more damaging to neurones than is O_2 lack alone (see Chapter 1, Section 11).

Another aspect of possible membrane damage following hypoxia is the liklihood of lipid peroxidation. Phospholipids have been shown to be substrates for lipid peroxidation in rat brain with phosphatidylcholine being the most active substrate (Sharma, 1977). Potent free radicals are normal by-products of mitochondrial oxidation. Under normal conditions however, the structural integrity of the mitochondria protects the membrane phospholipids from auto-oxidation by preventing the interaction between substrates and soluble pro-oxidant factors. Other protective mechanisms are enzymes such as superoxide dismutase, catalases and peroxidases along with various antioxidants such as ascorbic acid, tocopherols and thiol containing compounds. Catecholamines and 5-HT have been shown to inhibit lipid peroxidation in rat liver mitochondria and microsomes (Schaefer *et al.*, 1975) and maybe are capable of the same reactions in brain.

There is evidence that when brain tissues are incubated *in vitro* in the presence of O_2 and suitable free radical initiators, appreciable amounts of lipid peroxides are formed (Westerberg *et al.*, 1979). Selective changes in brain tissue phospholipids and fatty acids occurred during peroxidation *in vitro* with a fall in phosphatidylethanolamine, no change in phosphatidylcholine, and a decrease in arachidonic and docosahexaenoic acids. As yet however there is no evidence that a reduction of brain tissue P_{O_2} may enhance the formation

of free radicals and thereby induce lipid peroxidation (Folbergrová et al., 1979).

Should lipid peroxidation occur in vivo under conditions of hypoxia it will have important implications for prostaglandin synthesis. Firstly, lipid peroxidation has been shown to cause a decrease in arachidonic acid in vitro (Westerberg et al., 1979) and as already described, arachidonic acid is an essential substrate for the production of prostaglandins, thromboxanes and prostacyclin (see Chapter 1, Section 11.2.). Secondly, cyclo-oxygenase, which catalyzes the oxygenation of arachidonic acid, can be stimulated by a wide variety of peroxides such as its own product, PGG_2, an endoperoxide-hydroperoxide, as well as lipid peroxides (Hemler et al., 1979; Lands, 1979).

The possible involvement of phosphatidylinositol in synaptic transmission has already been discussed, but what of the other main lipid classes? Sen & Cooper (1978) proposed that disruption of the membrane phospholipids by phospholipase activity can lead to depolarization of synaptosomes which promotes both transmitter release and inhibition of the energy-dependent high affinity choline uptake system. Similarly Kunze (1973) demonstrated that the deacylation of phosphatidylethanolamine was increased in the presence of 0.1mM ACh with 0.1mM neostigmine, 1mM NA, and 1mM 5-HT, presumably by stimulating both phospholipase A_1 and A_2. NA, 5-HT and adrenaline have also been shown to increase the concentration of FFA in synaptosomes (Price & Rowe, 1972), and it may be that these changes reflect changes in the structure and properties of synaptic membranes during synaptic transmission. Another interesting relationship between neurotransmitters and membrane composition was shown by Asakawa et al. (1978). These authors found that the guanylate cyclase of rat synaptic plasma membranes was remarkably stimulated by several unsaturated fatty acids, whereas

saturated fatty acids had little effect. For example, arachidonic acid stimulated guanylate cyclase 5.8-fold at 0.4mM, and oleic acid 5.9-fold at 1.2mM. Could it be possible that the 80% increase in cyclic GMP which accompanies hypoxia, so mild that there were no significant changes in cerebral lactate (Gibson et al., 1978), is caused by fatty acid stimulation of guanylate cyclase?

Let us now go on to consider the ways in which hypoxia is likely to influence mitochondrial function and whether any clues can be found to help understand just why synaptic transmission is more vulnerable to O_2 lack than is axonal conduction. Once again there have been many studies undertaken to look at mitochondrial function in vitro either in anoxic conditions or after isolation from ischaemic brains.

It is very unlikely that mitochondrial respiration is directly affected by a reduction in O_2 availability due to the very low apparent K_m for O_2 of 0.1μM that has been determined in rat brain mitochondria (Clark et al., 1976). Contrast this with the values of 0.5mM and 1.0mM that have been determined for cerebral tyrosine hydroxylase and tryptophan hydroxylase respectively (Fisher & Kaufman, 1972; Kaufman, 1974), and the value of 4μM which has been found to be the critical O_2 concentration for synaptosomal O_2 consumption (Chapter 2, Section 7.2.3.).

It seems more probable that mitochondrial function will be influenced indirectly by any reduction in P_{O_2}. The usual method for measuring mitochondrial integrity is to calculate the respiratory control index (RCI) as defined by Chance and Williams (1955). Calculation of the RCI in vitro involves the stimulation of mitochondrial respiration to a maximum rate by the addition of ADP but it would seem unlikely that mitochondria in vivo respire maximally. However a fall in the RCI does seem to correlate well with a decline in mitochondrial

function (Chefurka, 1966). This discussion will centre on three experiments describing the effects of severe O_2 deprivation on mitochondria.

(i) Mitochondria isolated from ischaemic brains show a fall in the RCI, decreased ATP synthesis and a fall in respiration due principally to a decrease in State 3 respiration (Kuwashima et al., 1978; Rehncrona et al., 1979).

(ii) Incubation of an isolated retina produces mitochondrial swelling 10 min after withdrawal of O_2 and after only 3 min if both O_2 and glucose are withheld (Webster & Ames, 1965).

(iii) Anoxic incubation of mitochondria produces a loss of RCI and a fall in the ADP/O ratio (Markelonis & Garbus, 1975). These same mitochondria rendered anoxic in a polarographic medium show an accumulation of FFAs - particularly arachidonic, stearic and palmitic acids - similar to that seen with "aged" mitochondria or mitochondria isolated from ischaemic tissue.

Considering first point (ii), cerebral mitochondria have been found to show a non-uniform morphologic response to osmolar changes (Holtzman et al., 1978). Hypo-osmolarity leads to inhibition of energy metabolism in brain, with inhibition of isolated brain mitochondrial phosphorylation-coupled respiration and a fall in glycolysis and high energy phosphate concentration in brain slices. Hypo-osmolarity also reduces synaptosomal O_2 consumption (Table 6.2.6.). In contrast, hyper-osmolarity produces uncoupling in mitochondria and increased glycolysis in brain slices. The end result is however that both hypo-osmolarity and hyper-osmolarity decrease the RCI of mitochondria - hypo-osmolarity by decreasing State 3 and hyper-osmolarity by increasing State 4 respiration.

A connection between mitochondrial swelling (Webster & Ames, 1965) and brain swelling in ischaemia is of interest here. Using a

stroke model in baboons, Symon (1978) suggested that an area of structural loss in stroke (ischaemia) was probably surrounded, for some time in the acute phase, by an area of functional neuronal suppression in which the structural integrity of the neurones was immediately and even permanently preserved. This region was termed the "ischaemic penumbra". The differential failure of neuronal metabolic processes such that in the penumbral area, synaptic transmission is impaired but the energy state and the ionic balance - as measured by K^+ sensitive microelectrodes - are maintained at normal levels. K^+ loss only occurs when CBF is lower than levels for failure of electrical function. These results are very similar to those found in hypoxia where there are neuronal alterations before any significant changes in energy state provided that the CBF is well maintained (Duffy et al., 1972 ; MacMillan & Siesjö, 1972; Bachelard et al., 1974). A more significant degree of ischaemia in the brain will result in an irreversible loss of function in certain finite areas of tissue, surrounded by a penumbral zone in which though tissue function is lost, tissue structure is maintained (Symon, 1978). It may well be that the correspondence of the level of ischaemia necessary to evoke brain swelling, and the approximate level necessary to abolish brain function, may indicate an increase in osmolarity.

Points (i) and (iii) may now be considered in relation to the release of FFAs by mitochondria when deprived of O_2 (Markelonis & Garbus, 1975). I have already discussed in the previous Section the relationship between mitochondrial function and FFAs with reference to their possible influence on synaptosomal O_2 consumption, therefore only a brief description will follow. FFAs were identified as the agents in microsomes responsible for stimulating the latent mitochondrial ATPase and respiration of rat liver mitochondria (Pressman & Lardy, 1956). Long-chain unsaturated FFAs are more effective here than saturated fatty

acids (Borst et al., 1962; Chefurka & Dumas, 1966). Although one would expect FFAs to stimulate respiration by acting as uncouplers, the opposite effect is often observed although the RCI is always reduced on "ageing" of mitochondria. Exogenous oleic acid inhibited the respiratory activity of brain mitochondria and decreased ATP synthesis (Lazarewicz et al., 1972; Kuwashima et al., 1976). Replacing respiratory cofactors such as NAD^+, CoA and cytochrome c restores mitochondrial oxidation (Bjorntorp et al., 1964; Lochner et al., 1976).

Finally, since FFAs have a detergent-like character, it is reasonable to assume that they might affect the structure of mitochondrial membranes, thus releasing membrane-bound enzymes. At low concentrations (0.01-0.1mM) fatty acids, such as oleic and palmitic acid, increased the specific hexokinase activity in the supernatant fraction of mitochondria (Dománska-Janik et al., 1978). This result was solely due to a solubilizing effect rather than an effect on membrane structure since fatty acid concentrations greater than 0.1mM were required to produce membrane damage. Dománska-Janik et al. (1978) have pointed out that the observed rise of hexokinase specific activity in brain cytoplasm under some hypoxic conditions may partly correspond to the sensitivity of the mitochondrial-bound enzyme towards the solubilizing action of fatty acids. In the same context ischaemia, but not hypoxia, results in a significant decrease in membrane-bound glucose-6-phosphatase activity in the microsomal fraction, with a concomitant increase in the cytosol of guinea-pig cerebral cortex (Rossowska & Dabrowiecki, 1978). These changes in glucose-6-phosphatase distrubution were accompanied by a fall in phosphatidylcholine and phosphatidylethanolamine content of the brain. It was concluded here that ischaemia activates endogenous membrane-bound phospholipases which were probably responsible for the release of membrane-bound protein components into the cytosol.

The activity of phospholipases and the release of FFAs are in fact intimately connected. The work of Gan-Elepano & Mead (1978) suggests that the normal first step in the alteration of membrane lipid acids in cerebral mitochondria and microsomes is their release to other membrane-bound enzyme systems by a membrane-bound phospholipase A. It is the mismatch between the deacylation reactions of phospholipases and the energy-dependent reacylation reactions that is responsible for the accumulation of FFAs in anoxia and ischaemia. This accumulation of FFAs may in turn lead to increased activation of the phospholipases. In relation to anoxic mitochondria Markelonis & Garbus (1975) suggested that anoxia leads to the release of sequestered Ca^{2+} which activates phospholipases to liberate FFAs. Since FFA release is likely to affect membrane structure it is reasonable to assume that a further imbalance in Ca^{2+} distribution will occur leading to perpetuation of the cycle.

Finally one must consider the process of neurotransmission since much of the discussion so far can apply to the whole of the neurone whereas it is the nerve-ending and synaptic transmission which is most probably directly affected by hypoxia. When considering neurotransmitter metabolism (e.g. synthesis, release, re-uptake, degradation) one must keep in mind that hypoxia alone does not affect the energy status of the brain and that the intact blood supply does not limit the availability of neurotransmitter substrates/precursors. The principle neurotransmitters which have been studied in hypoxia are (a) acetylcholine, (b) GABA and (c) DA, NA and 5-HT. (See also Chapter 1, Section 8.4.).

(a) Acetylcholine

ACh synthesis has been found to be extremely sensitive to hypoxia with a 43% decrease in the synthesis of Ach from [U-^{14}C]-

glucose in hypoxic conditions which were so mild that there were no significant changes in cerebral lactate (Gibson et al., 1978). Hypoxia also reduces, to a lesser extent, the incorporation of $[^2H_4]$-choline into ACh and this is not due to an inhibition of choline uptake because hypoxia also increases the cerebral concentration of choline (Gibson & Blass, 1976). The total ACh falls only in severe hypoxia suggesting that a small pool of ACh with a high turnover rate is affected although the localization of this pool is unknown. Although carbohydrate oxidation is one of the main factors on which the synthesis of ACh depends closely in mouse and rat brain, the change in ACh synthesis cannot be accounted for by a change in glucose utilization (Gibson et al., 1978).

(b) γ-aminobutyric acid

GABA has an important role to play in the brain both as an energy substrate and as an inhibitory neurotransmitter. When Wood (1967) discovered a marked increase in cerebral GABA after rats had been kept for 10 min at 8% O_2 it was thought that GABA acting as an inhibitory neurotransmitter, depressed nerve transmission to conserve the available high energy compounds. This was an attractive hypothesis because increased cerebral GABA levels were found in a wide range of hypoxic animals at a critical level of 7-8% O_2 (Wood et al., 1968). These results compared well with the data of Gurdjian et al. (1949) who had discovered a critical O_2 concentration of 8% for changes in lactate and PCr. Unfortunately this hypothesis falls down when considering the time factor involved. Neuronal silence occurs in hypoxia within 1-2 min (Erdmann et al., 1973) whereas the accumulation of GABA takes at least 15 min to increase by 13% of normal (Wood et al., 1968). The accumulation of GABA could be due to the decreased utilization of GABA via the GABA shunt pathway of the TCA cycle accompanied by the continued production of GABA by anaerobic glutamic acid decarboxylase.

(c) Dopamine, noradrenaline and 5-hydroxytryptamine

Of the neurotransmitter systems mentioned so far, those involving DA, NA and 5-HT are most likely to be affected by hypoxia because of their requirement for O_2 as a cofactor in their synthesis. The steps in the synthesis and metabolism of these monoamines which require molecular O_2 are:

tryptophan	tryptophan hydroxylase →	5-hydroxytryptophan (5-HTP)
tyrosine	tyrosine hydroxylase →	3,4-dihydroxyphenylalanine (DOPA)
dopamine	dopamine β-hydroxylase →	noradrenaline
monoamines	monoamine oxidase →	aldehydes

Both tryptophan and tyrosine hydroxylase are rate-limiting enzymes in the synthesis of 5-HT and DA and their apparent K_m values for O_2 are 1mM and 0.5mM respectively. Calculation of these K_m values depends to a large extent on the type of pteridine cofactor used in the assay since the use of the artificial cofactor 6,7-dimethyltetrahydropterin ($DMPH_4$) will give a higher apparent K_m value than the natural cofactor tetrahydrobiopterin (BH_4), (Friedman et al., 1972).

A rough estimate of turnover rates indicated that <0.1% of O_2 consumed by the brain is utilized in the direct synthesis and metabolism of NA, DA and 5-HT (Davis & Carlsson, 1973b). The hydroxylase enzymes are therefore poor competitors for cellular O_2 with mitochondria and provided that synthesis and degradation are retarded to a similar degree in hypoxia, the absolute brain monoamine levels will remain unchanged (Brown et al., 1975).

Hydroxylation rates have been shown to be reduced in hypoxia before there are any changes in the adenylate energy charge (Carlsson, 1978) and decreased 5-HTP is found before any increase in cerebral

lactate (Davis & Carlsson, 1973a). What is most interesting though is the possible relationship between the disturbed monoamine metabolism and the behavioural effects observed in mild hypoxia. DA and NA neurones appear to show differences in susceptibility to anoxia. 24 hr after ligation of the gerbil carotid artery one finds a 46% fall in DA but no change in NA content on the infarcted side (Zervas et al., 1974). Carlsson (1978) has also suggested that hypoxia inhibits the firing of dopaminergic neurones and enhances the firing of noradrenergic neurones. The inhibitory effect of hypoxia on DA turnover was particularly prominent and was suggested to be caused by inhibition of nerve impulse-induced neurotransmitter release. Hypoxia has been found to disrupt the reversibly conditioned avoidance response (CAR) in rats - an effect which can be reversed by treating the animals with DOPA (Brown et al., 1975). Since CAR is known to depend on intact catecholamine systems in the brain, these observations suggest that inhibition of catecholaminergic, especially dopaminergic activity is involved in hypoxia-induced CAR disruption. It is possible that this is a regulatory mechanism which is important for the behavioural inhibition and the preservation of energy in hypoxia (Carlsson, 1978).

Should there also be any increase in prostaglandin synthesis in hypoxia then it is likely that noradrenergic neurones will be affected since there is mounting evidence that prostaglandins of the E-series inhibit NA release by interfering with the availability of Ca^{2+} for stimulus-secretion coupling (Hedqvist, 1978).

To conclude this section I shall briefly summarize the effects of anoxia and hypoxia on cerebral metabolism as I have found for rat brain synaptosomes and then follow this by pointing out the reactions which are most likely responsible for the failure of neuronal activity in hypoxia.

(a) A critical O_2 concentration of approximately 4μM was found for the process of O_2 uptake by synaptosomes. A similar value was obtained regardless of the initial P_{O_2}.

(b) Synaptosomal O_2 consumption in conditions of low P_{O_2} (taken here as an O_2 concentration less than 14μM and above 4μM) was similar to that in fully aerated medium, i.e. they responded to the presence of glucose and BSA by increasing the rate of O_2 uptake.

(c) Glucose and BSA stimulate synaptosomal O_2 consumption by independent means such that one can predict the response of the two added together.

(d) Glucose stimulates synaptosomal O_2 consumption presumably by acting as a respiratory substrate, e.g. although measurement of lactic acid produced inconclusive results, the presence of glucose significantly reduced the pH of the synaptosomal suspension after 10 min incubation in normoxia, hypoxia and anoxia.

(e) BSA stimulates synaptosomal O_2 consumption when the tissue to BSA ratio is low, e.g. 1.45:10.

(f) BSA might possibly function by binding Ca^{2+} as a Ca^{2+}-free medium was found to stimulate both synaptosomal O_2 consumption and glycolysis to a similar extent.

(g) There is no evidence that BSA stimulates synaptosomal O_2 consumption by binding FFAs and thus preventing any adverse effects of FFAs. BSA does not contribute fatty acids to simulate synaptosomal O_2 consumption since a preparation of fatty acid-free BSA produces identical responses.

(h) Although an exact K_m value cannot be determined from the data presented here, an approximate value for glucose-supported synaptosomal O_2 consumption can be calculated as approximately 4μM taking the substrate (O_2) concentration at half maximum velocity.

(i) Synaptosomes can be incubated at an O_2 concentration of 140μM

for 30 min and at 30°C without any evidence of membrane disruption as measured by the absence of any increased activity of free lactate dehydrogenase - a cytoplasmic enzyme.

(j) Pre-incubation of synaptosomes did not increase respiratory activity and cannot be used to prevent the natural decline in the rate of O_2 uptake in the absence of glucose observed after storing the tissue on ice.

(k) If pre-incubation must be used, several precautions should be noted. (1) Pre-incubation in a shaking water bath preserves the response of synaptosomes to glucose and BSA although all the rates are reduced. The presence of glucose - but not BSA at the concentration used - during the pre-incubation will help prevent the fall in the rate of O_2 presumably by maintaining the levels of glutamine, ATP and PCr. (2) Pre-incubation using a non-shaking water bath should be avoided since synaptosomes will no longer respond to glucose and BSA will inhibit rather than stimulate O_2 consumption. Here also there is no advantage for adding glucose during the pre-incubation. (3) Both pre-incubation procedures (35 min at 30°C) produce a degree of membrane damage as measured by the increased activity of free lactate dehydrogenase. There was however no difference in lactate dehydrogenase activity to account for the different effects on synaptosomal O_2 consumption. (4) The Na_2HPO_4 concentration in the medium must be 20mM to prevent pre-incubation producing even more severe reductions in the rate of synaptosomal O_2 uptake.

(l) Synaptosomes were found to contain free fatty acids, the principal species were palmitic, stearic, oleic, linoleic and arachidonic acids. A 10 min incubation at 30°C in normoxic conditions produced a yield of 64µg FFA/mg protein - the main contribution coming from the long-chain unsaturated fatty acids.

(m) The yield of FFAs was not increased after hypoxic or anoxic incubation.

(n) The pattern of distribution of FFAs was similar in both the synaptosome fraction and the medium after filtration of the synaptosomal suspension.

Extrapolation of these results to the in vivo situation poses one important problem - what P_{O_2} value truely represents hypoxia in cerebral tissues in vivo? A value of 20-25mmHg for P_{AO_2} has been described as the level of O_2 lack that can be tolerated in experimental animals before there are any significant changes in cerebral concentrations of ATP, ADP and AMP (Duffy et al., 1972; MacMillan & Siesjö, 1972; Bachelard et al., 1974). However brain P_{O_2} as measured by microelectrodes gives a range of 1-95mmHg (Bicher et al., 1971). Similarly simultaneous measurement of action potential frequency and P_{O_2} during transient hypoxia (1 min) in rat brain indicated that in tissue areas with a high initial P_{O_2}, the critical P_{O_2} value could be as high as 20mmHg compared to < 0.5mmHg in regions with low P_{O_2} (Erdmann et al., 1973). The critical P_{O_2} here is defined as that producing a fall in action potential rate to < 10/min.

In the experiments described above using synaptosomes as the experimental tissue, hypoxic conditions have been taken as a P_{O_2} of 10mmHg and less. Although the critical P_{O_2} for synaptosomal O_2 consumption was determined as approximately 3mmHg, it was not possible practically to commence or maintain incubations at this low level of O_2.

What can be concluded from these results in relation to neuronal failure in hypoxia? Synaptosomal O_2 consumption has been shown to be very sensitive to the external environment although the critical O_2 concentration of 4μM is remarkably constant. Maximal rates of O_2 uptake over long time periods require a medium such as Krebs phosphate medium with a Na_2HPO_4 concentration of at least 20mM supplemented with 10mM glucose as also suggested by Bradford (1969). Since hypoxia is not

associated with changes in the energy status of the cells, then one would expect nerve-endings in vivo to be unaffected provided that the CBF is well maintained. Any change in osmotic conditions will alter synaptosomal O_2 consumption, for example, hypo-osmotic conditions reduce the rate of O_2 uptake. The results of experiments using BSA are inconclusive at the present time however the most likely reason for BSA stimulating synaptosomal O_2 uptake to such a significant degree in hypoxia is its binding of Ca^{2+}. Neither hypoxic or anoxic incubation (10 min) produced an increased yield or altered distribution of FFAs from synaptosomes. Either nerve-endings are not the source of FFAs known to be released by both whole brain and isolated mitochondria in ischaemia (Bazán, 1970; Lazarewicz et al., 1972), or my experimental design requires modification.

Using information from the literature on hypoxia, the most probable reason for the inhibited neuronal activity is the effect of O_2 lack on neurotransmitter synthesis. Consideration of the K_m values for O_2 of tyrosine and tryptophan hydroxylases shows that they are likely to be affected before the fall in O_2 consumption by synaptosomes and before mitochondrial respiration is affected. This would support the evidence that there are behavioural and neuronal alterations before any significant changes in ATP, ADP and AMP because synaptosomal O_2 consumption and therefore the production of ATP from oxidative phosphorylation is unchanged at degrees of hypoxia known to affect the synthesis of DA and 5-HT. Of these two neurotransmitters, DA is the most likely candidate responsible for the behavioural inhibition and the preservation of energy in hypoxia.

As for the more profound degree of O_2 lack experienced in anoxia and ischaemia, the metabolic and structural alterations (see Chapter 1, Sections 7, 9 and 10) can all be traced to the derangement

in energy production. Of particular importance in determining the extent of recovery is likely to be the destruction of membranes by phospholipase activity. Not only will this lead to the accumulation of FFAs but will consequently also stimulate lipid peroxidation and both in turn will influence the synthesis of potent molecules such as prostaglandins, thromboxanes and prostacyclin.

Whether or not the selective breakdown of phosphatidylinositol occurs also in hypoxia is unknown but this would serve two functions since it has been suggested that phosphatidylinositol is involved in synaptic transmission and one of the chief breakdown products is arachidonic acid.

3. IS THERE AN O_2 SENSOR IN CEREBRAL TISSUES?

Currently the existence of an O_2 sensor within the brain is purely speculative and because absence of evidence is not necessarily evidence of absence, the search continues. Possible candidates for the role of O_2 sensor have already been described in Chapter 1, Section 5 and three of these will be discussed further in relation to the biochemical changes occurring in hypoxia.

Firstly let us consider the mitochondrial respiratory chain. Changes in the oxidation-reduction level of the terminal oxidase and of members of the chain near it occur at O_2 concentrations considerably exceeding the critical level based upon respiratory activity (Chance, 1957). The rate of O_2 consumption by the respiratory chain will depend on the concentrations of cyt a_3 and O_2 such that when the P_{O_2} falls to levels that are no longer in excess of the needs, the rate of respiration can be prevented from falling as a reduction in P_{O_2} is compensated for by a rise in the concentration of reduced cyt a_3. An excess of the terminal oxidase would allow a sufficient amount of reduced form to remain and carry on the respiratory process without a measurable change in the respiratory rate. This protective, cushioning effect of the cytochrome chain may explain the failure of certain physiological functions at P_{O_2} exceeding the critical value for mitochondrial respiration but can the altered redox state of cyt a_3 be used as an O_2 sensor? Because cyt a_3 becomes gradually more reduced as O_2 becomes scarcer, monitoring of cyt a_3 would be a very sensitive means of telling when a tissue enters a hypoxic state before respiration falls dramatically. Although this is an attractive hypothesis, the precise redox state of cyt a_3 in brain tissue <u>in vivo</u> has not yet been confirmed (Siesjö, 1978) and Jöbsis (1977) in a discussion on a molecular O_2 sensor suggested that there was currently little evidence to support this hypothesis.

Secondly the role of O_2 sensor could be assigned to an oxidase with a relatively low affinity for O_2, compared to for example, the mitochondrial respiratory chain. In this system signs of hypoxia would occur at a correspondingly higher P_{O_2} and therefore provide a larger safety margin for cerebral function. As discussed in the previous section, both tyrosine and tryptophan hydroxylases have relatively high K_m values for O_2 and therefore low affinities. The rate of synthesis of DA, NA and 5-HT all depend on these enzymes but their overall levels remain unchanged in hypoxia due to simultaneous inhibition of the degradative enzymes. However, as in most biological systems, it is likely that the change in rate of synthesis will be the factor that is registered rather than absolute levels. The most likely candidate of these three neurotransmitters is either DA or 5-HT. Changes in both tyrosine and tryptophan hydroxylase activities occur at degrees of hypoxia too mild to significantly alter ATP, ADP and AMP and decreased 5-HTP is found before any increase in cerebral lactate (Davis & Carlsson, 1973a). The firing of dopaminergic neurones has been suggested to be selectively inhibited in hypoxia although the hydroxylation of tyrosine is somewhat less strikingly influenced by varying P_{O_2} than that of tryptophan (Carlsson, 1978). Before either of these can be considered further as candidates for the role of O_2 sensor, further studies must be made to determine the time response factor in the influence of varying P_{O_2}.

Finally there is the possibility of membrane lipid changes. In particular there is the possibility of hypoxic-induced breakdown of phospholipids which could lead to increased synthesis of prostaglandins to act as messengers to the local environment of changes in P_{O_2}. Much more research has yet to be done on this particular hypothesis and indeed all three ideas require further experimentation. In fact the final conclusion may be that there are two types of sensing mechanism, one to act

locally and protect metabolism whether in a region of normally high or low P_{O_2}, and another to act on a more generalized level.

FURTHER WORK

There still remain many unanswered questions as to the degree of dependence of cerebral metabolism and function on O_2 availability. In particular, experiments need to be conducted in hypoxic conditions which tend to have been neglected in the literature to date.

Deriving from the experiments described in Chapter 2 is the still unsolved problem of the possible influence of FFAs on synaptosomal O_2 consumption. It would be interesting to test the effect of varying amounts of exogenous long-chain fatty acids - both saturated and unsaturated - on the rate of synaptosomal O_2 uptake in media of both high and low O_2 content. Controls for these experiments should consist of tissue stored on ice, i.e. no pre-incubation, and using a Krebs phosphate medium supplemented with both glucose and glutamine.

The use of an oxystat is likely to provide the best conditions for studying the O_2 dependence of synaptosomes. The oxystat described in Chapter 2, Section 5.2. is not suitable for incubations at P_{O_2} values equivalent to the critical O_2 concentration of 4μM as determined for the process of synaptosomal O_2 consumption. An automated design such as that of Jones & Mason (1978) is probably more suitable. Similarly, improvements in the rapid filtration of synaptosomes and incubation medium will be required to cope with larger tissue concentrations.

Firstly the oxystat should be used to calculate a more accurate determination of the apparent K_m for O_2 for synaptosomal O_2 consumption. Once this has been measured, it can be used to establish, along with the critical O_2 concentration, a closer approximation of P_{O_2} likely to be equivalent to hypoxia *in vivo*. Following from this, the oxystat can be set to incubate synaptosomes in normoxic, hypoxic and anoxic conditions, and the following substances monitored:

(i) The adenylate energy charge and PCr levels.

(ii) Effects on Ca^{2+} distribution.

(iii) The phospholipid composition of synaptosomes - there may be a selective loss of one particular class of phospholipid such as phosphatidylinositol.

(iv) The accumulation and distribution of FFAs with particular reference to the proportion of unsaturated to saturated long-chain fatty acids.

(v) If there are any marked changes in arachidonic acid levels, one could consider measuring prostaglandin, thromboxane and prostaglandin synthesis.

(vi) The activity of phospholipases and their dependence on Ca^{2+} if any changes in Ca^{2+} distribution can be measured.

(vii) The activities of tyrosine hydroxylase and tryptophan hydroxylase with particular reference to the time-dependency of altered activity due to O_2 lack.

Determination of any of these factors would help in consideration of the questions posed in Chapter 3 and should help in our understanding of the seemingly anomalous situation arising in hypoxia with regard to neuronal function. Similarly any disturbance of synaptosomal metabolism in anoxia might provide information as to the efficient handling of patients suffering from acute O_2 insufficiency and particularly, those afflicted by recurrent attacks of cerebral infarction.

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